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OPSONIZING AND BACTERICIDAL EFFECTS OF SERA FROM GNOTOBIOTIC AND CONVENTIONALIZED RATS ON ^{32}P-LABELLED *E COLI*

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Using an in vitro system based upon radioisotope technique th opsonizing and bactericidal effects upon *E coli* of sera from germfree *E coli* and *B subtilis* monocontaminated and conventionalized rats were investigated Sera from the germfree animals were found to possess opsonizing as well as bacteriotoxic properties The opsonizing activity was highest in sera from rats monocontaminated with *E coli* and lowest in sera from germfree animals The bactericidal activity was lowest in sera from germfree rats Specific and unspecific monocontaminations as well as conventionalization increased the serum bactericidal activity against the strain of *E coli* used

Serum components play important roles in the defence of the host against invading bacteria Serum is generally required for the effective ingestion of most bacteria by phagocytes (7) More or less specific antibodies and probably also complement components participate in the opsonization of *E coli* (7) Apart from the phagocytic response serum components also exert a bactericidal effect upon such bacteria in vitro and probably also in vivo (5 12)

These two factors in host defence might be influenced by previous exposure of the host to microbes with a certain similarity in antigenic properties Sera from gnotobiotic animals appear well suited for studying the degree of specificity involved in the opsonizing and bacteriotoxic systems of serum

By means of radioisotope technique an in vitro system has been developed which has been used for the quantitative determination of the opsonizing and bacteriotoxic effects of serum from conventional rats on ^{32}P labelled *E coli* (12) In the present investigation these effects were studied in sera taken from germfree monocontaminated and conventionalized rats

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MATERIALS AND METHODS

Animals

Germfree rats of the CDF strain (Charles River Breeding Lab Wilmington Mass USA) were born and raised to the age of 40-50 days in germfree conditions whereafter they were divided at random into the following four experimental groups a) germfree (GF) b) monocontaminated with *Escherichia coli* (MEC) c) monocontaminated with *Bacillus subtilis* (MBS) and d) conventionalized (CONV) Each group consisted of five rats without consideration to sex difference

The germfree and monocon-

germfree animals were kept outside the isolators in an ordinary animal room

Germfree Technique

The animals were housed in flexible film isolators (Snyder Manufacturing Co Inc New Philadelphia Ohio USA) sterilized by peracetic acid The fiberglass air inlet filter was sterilized at 150 C for 12 hours The oil air-outlet filter was sterilized by adding 5 per cent Dowicide 9 (Dow Chem Co Midland Mich USA) to light mineral oil

Diet

All animal were fed on commercial rat food (5) and water *ad libitum* In addition a mixture of 2 5 g Meritene (The Dietene Co Minneapolis Minn USA) 2 5 mg ascorbic acid and 0 5 mg vitamin K_1 per 100 ml of water were given freely The dietary compounds were autoclaved separately at 120 C for 20 min

Bacteriological Control

Swabs from the inner surface of the isolators and fecal samples were tested weekly for sterility by anaerobic incubations in Fluid Thioglycollate medium (Difco) and aerobic incubations in Dextrose broth (Difco) and on blood agar plates All incubations took place both at room temperature and at 37 C In addition, samples were cultivated at 28 C on Sabouraud Dextrose agar (Difco)

Samples from the monocontaminated isolators were controlled similarly

Bacterial Strain

The strain of *E coli* used for monocontamination of the germfree rats was identical with the one employed in the phagocytic system (12) The ...n was originally isolated from feces of a con...ional rat of a local strain

...an unrelated monocontaminating organism ... used a strain of aerobic Gram positive spore ...ming rods picked up at random from rat feces and identified by conventional methods as a member of the species *Bacillus subtilis*

Contamination of Germfree Rats

The bacterial strains were grown in a casein medium (1) at 37 C for 18 hours Aliquots of the cultures were transferred into sterile ampules and put into the isolators through the entry port Each animal received 1–2 ml of the cultures *per os* and 1–2 ml was swabbed on the skin of the animals which were kept in the isolators for 60 days

The germfree animals to be conventionalized were taken out of the isolators and exposed to conventional visitor rats

Serum

The germfree and monocontaminated rats were taken out of the isolators and immediately bled by heart puncture Serum from each rat was collected and stored as individual samples (12)

Determinations of Phagocytosis and Release of ³²P into the Medium

Polymorphonuclear leucocytes (PMN) were obtained from the peritoneal cavity of conventional rats of a local strain (12) Monolayers of PMN were prepared in tissue culture tubes to which were added ³²P labelled *E coli* (10^9 bacteria/ml) suspended in Krebs Ringer phosphate buffer with 10mM glucose (KRG) and 10 per cent of each serum sample to be tested The tubes were incubated for 15 min at 37 C whereafter phagocytosis was determined by measuring the incorporation of ³²P into the PMN as described (12)

In order to measure the bactericidal effect of the serum samples the release of label from the bacteria into the medium was determined as proposed by *Spitznagel & Wilson* (10) The microbial suspension in KRG with 10 per cent of serum was incubated at 37 C for 15 min centrifuged at 6000 × g/10 min/0 C and radioactivity determined in the supernatant (12)

Statistical Analysis

Statistical analysis was carried out by means of the two sample ranks test of *Wilcoxon White* (3 11)

RESULTS

Phagocytosis

Fig 1 illustrates the rate of incorporation into PMN of ³²P labelled *E coli* in KRG with and without 10 per cent of serum from germfree rats (GF serum) The incorporation into PMN in the medium containing GF serum was about ten times as high as that obtained in KRG alone

In Fig 2 is demonstrated the rate of incorporation into PMN of ³²P labelled *E coli* suspended into media containing serum from the various groups of rats The incorporation was least when the medium contained 10 per cent of GF serum The incorporation of labelled bacteria into PMN in KRG with serum from the *E coli* contaminated rats (MEC serum) was higher than that obtained in KRG with GF serum ($p < 0.01$) A slightly, although not

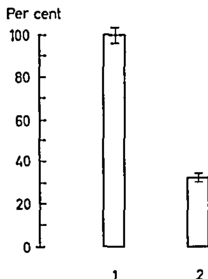


Fig 3 Release of label from ^{32}P labelled *E coli* incubated in KRG with/without GF serum A typical experiment which illustrates the released radioactivity when GF serum pooled from five rats was present or absent in the incubation medium Aliquots (2 5 ml) of a suspension of ^{32}P labelled *E coli* (10^9 per ml) were incubated at 37 C for 15 min in quadruplets The suspending media contained 1) KRG with 10 per cent GF serum 2) KRG Each column illustrates the mean release of label in five observations ± standard deviation expressed as per cent of the release in medium 1

DISCUSSION

The results presented indicate that the opsonic system which has been shown to enhance PMN phagocytosis of *E coli* (12), is functionally present in serum from germfree rats The results are in agreement with previous findings concerning the existence of opsonins involved in Kupffer cell phagocytosis of colloidal gold in serum from germfree rats (8) and the presence of opsonins stimulating clearance of goat erythrocytes from the circulation in germfree chickens (9)

It is generally presumed that germfree animals are less exposed to antigenic stimuli than their conventional counterparts (2) As suggested by *Saba et al* (8), the presence of opsonic activity in sera from germfree animals might indicate that some of the factors involved in opsonization are represented by so called natural antibodies The food used for raising germfree animals may contain various types and numbers of microorganisms Although killed by sterilization before being introduced into the isolators, these microbes might serve as antigenic stimuli in germfree animals (2) and might account for the production of antibodies (4)

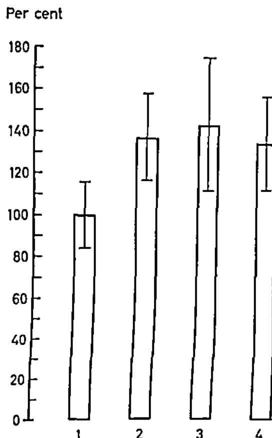


Fig 4 Release of ^{32}P from labelled *E coli* incubated in media containing sera from germfree and exgermfree rats Aliquots (2 5 ml) of a suspension of ^{32}P labelled *E coli* (10^9 per ml) in the medium to be tested were incubated at 37 C for 15 min in quadruplets The media contained KRG with 10 per cent of either serum 1) GF 2) MEC 3) MBS and 4) CONV Each column represents the mean of 10 individual observations in two experiments ± standard deviation expressed as per cent of the release obtained in medium 1 which is set as 100 per cent

The results show that only monocontamination with *E coli* increases the opsonic activity of serum against *E coli* The apparently higher activity found in the MBS and CONV sera was not significantly differently from that of the GF sera Studies of larger groups

of monocontaminated and conventionalized animals might, however, yield more conclusive data concerning this aspect. It may be mentioned that the conventionalized rats were exposed to rats harboring the same flora as that present in the rat from which the test strain of *E coli* was isolated. *E coli* was found to be established in the intestinal tract of the conventionalized rats. However, the absence of a specific marker on the test strain excludes the possibilities of confirming the establishment in the intestinal tract of this particular strain. The observed differences between the MEC and CONV sera upon phagocytosis might therefore be due to different specificity in antigenic stimulation.

Previous studies have shown that the release of ^{32}P from *E coli* represents a convenient indirect measure of the bactericidal activity of the serum to be tested (10, 12). The present findings show that both specific and unspecific monocontaminations, i.e. contaminations with *E coli* and *B subtilis*, produce the same increase in the serum bactericidal activity against the *E coli* used.

It is generally accepted that serum components of both antibody and complement characters are involved both in the opsonization of *E coli* and in the humoral destruction of the same microbe (5, 7). The present results make it reasonable to assume that specific components are more important for PMN phagocytosis of *E coli* than for the humoral destruction of the same microbe. Further studies in order to elucidate this question are in progress.

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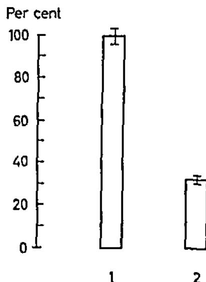


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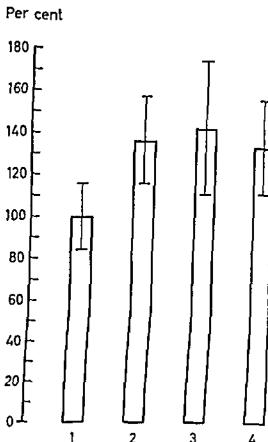


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recovered from the inoculated diphasic media at all Consequently, we lowered the concentration of MB in the medium to 0 0005 per cent

TABLE 1 *Isolation of* Mycoplasma pneumoniae *from 7 of 314 Hospitalized Patients*

Diagnosis	Number of patients		
	Age 0–14	Age ≧ 15	Total
Pneumonia	34(5)	128(2)	162(7)
Respiratory illness without pneumonia	49	35	84
No respiratory illness	20	48	68
Total	103	211	314

Figures in brackets indicate isolation of *M pneumoniae*

Table 2 shows the results obtained with the three different media used for a total of 353 isolation attempts in the last 229 patients Eight strains of *M pneumoniae* were isolated from seven of these

By primary inoculation onto agar medium *M pneumoniae* colonies were obtained from seven specimens the eighth strain was recovered only in broth without MB

The inoculation into broth with glucose and phenol red without MB resulted in acid formation due to *M pneumoniae* in three cases

None of the diphasic media inoculated the eight throat swabs showed any colour change as compared to uninoculated control media, but by transfer of seven of them to agar or broth without MB *M pneumoniae* was recovered in two cases

Of the seven patients from whom *M pneumoniae* was isolated one had received tetracycline for 3 days prior to isolation three patients had been treated with penicillin or sulphonamides, and three had not received any drugs before isolation of the agent

Serological Examinations

Antibodies to M pneumoniae A positive reaction was found in 14 patients namely in 13 of the group with pneumonia and in one control patient Both the FAT and the IHA tests were positive in 11 of the patients who also had a positive CA reaction Either the FAT or the IHA test was positive in the remaining three patients two of these including the control patient had a negative CA reaction and *M pneumoniae* was not isolated from either of them The third patient had a positive CA test and *M pneumoniae* was found in his throat A significant rise in titre of antibodies to *M pneumoniae* was found in 8 patients while 3 showed a fall in titre The maximum FAT titre was 2560 and the mean titre 454 while the corresponding values in the IHA test were 160 000 and 2406 respectively

CF tests with viral and TRIC antigens Serological indication of infection due to *Influenza virus Adenovirus* and *Ornithosis a*

TABLE 2 *Comparison of Three Different Media for the Isolation of* Mycoplasma pneumoniae *Eight Strains Were Isolated from 353 Throat Swabs*

Mycoplasma medium	Indication of growth in number of cultures	Comments
Agar	Colonies 7/8	The negative one was positive on culture in broth
Broth with glucose and phenol red	Colour change 3/8	
Diphasic with methylene blue	Colour change 0/8	On transfer of 7 to broth or agar two strains were recovered

TABLE 3 *Cold Agglutinin Reaction in Relation to Other Serological Reactions*

Serological reaction with antigen from		Number of patients examined		
		CA positive	CA negative	Total
M pneumoniae	positive	12	2	14
	negative	49	251	300
Influenza A	positive	12	25	37
	negative	48	223	271
Influenza B	positive	0	1	1
	negative	60	247	307
Influenza C	positive	2	1	3
	negative	59	247	305
Adenovirus	positive	3	4	7
	negative	57	244	301
TRIC agent	positive	0	7	7
	negative	60	242	302

gent was found in 55 patients A positive reaction with *Influenza A* antigen accounted for 37 of the cases representing 36 adults and one child

Two patients with positive anti *M pneumoniae* tests also showed positive *Adenovirus* CF tests one of these showed a 16 fold rise in the FAT titre a 64 fold rise in the IHA titre and a four fold rise in the *Adenovirus* CF titre this patient also showed a positive Paul Bunnell test with a typical Davidsohn absorption result In the other patient no change in titre could be estimated due to his brief stay in hospital

Cold agglutinins A positive CA reaction was found in 61 patients (about 20 per cent of the total material) or in 42 of the 162 patients with pneumonia (26 per cent) The distribution in relation to other positive serological reactions is shown in Table 3 Out of 14 patients with a positive test for antibodies to *M pneumoniae* 12 also had a positive CA reaction This is a highly significant correlation ($p < 0.1$ per cent) when compared to 49 positive CA tests among 300 with a negative anti *M pneumoniae* test Among the 48 patients with a positive CF test for either *Influenza* or *Adenovirus* antibodies there were 17 with a positive CA reaction This correlation is significant ($p < 1$ per cent) on the background of 43 positive CA tests among 260 patients with negative CF tests for the viral antibodies

M pneumoniae *Infection*

Evidence of illness caused by *M pneumoniae* according to the criteria mentioned above was obtained in 12 patients in seven children between 2½ and 11 years old, and in five adults aged 17 19 20 31 and 63 years respectively All patients from whom *M pneumoniae* was isolated had a positive reaction for antibodies to that microorganism

The distribution of patients with *M pneumoniae* infection during the two-year period was remarkably uneven as is shown in Table 4 While the number of patients with pneumonia registered in the first year was about twice that of the second the percentage of patients with evidence of *M pneumoniae* infection was 2 per cent in the first and 18 per cent in the second year this difference is significant ($p \sim 0.1$ per cent)

Isolation of Other Mycoplasmas

As a by product of this study a total of 40 strains of *Mycoplasma* other than *M pneumoniae* were isolated (Table 5) They were recovered from 34 of the last 229 patients where broth and agar media without MB were employed and from one patient where

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TABLE 4 *Incidence of* M pneumoniae *Infection in Patients Hospitalized with Acute Respiratory Illness During a Two Year Period*

Diagnosis	Number of patients			
	23/3 1966 to 28/2 1967		1/3 1967 to 25/3 1968	
	M pneumoniae infection	Other aetiology	M pneumoniae infection	Other aetiology
Pneumonia	2 (2)	103	10 (18)	47
Respiratory illness without pneumonia	0	69	0	15
No respiratory illness	0	49	0	19
Total	2	221	10	81

Figures in brackets indicate *M pneumoniae* infections as the percentage of all pneumonias during the period

TABLF 5 *Isolation of Mycoplasma Species Other than* M pneumoniae *from 34 out of 229 Patients*

Age group	Patients (strains)				
	M salivarium	M orale type 1	M hominis	Non identif	Total
< 2					
2– 4		1 (2)			1 (2)
5–14	2*(2)	1 (1)			3 (3)
15–44	6 (8)	2§(3)	2 (2)	2 (2)	12 (15)
45–64	6 (7)		1 (1)		7 (8)
≥65	8 (8)	2 (3)	1 (1)		11 (12)
Total	22 (25)	6 (9)	4 (4)	2 (2)	34 (40)

* *M pneumoniae* was also isolated from one of these patients
§ In a 3rd patient the first throat swab yielded a *M orale* type 1 and the second a *M salivarium*

the diphasic medium with 0 001 per cent MB is used *M salivarium* was isolated from 22 ents one of whom also yielded a *M orale* type 1 The latter species was found in seven patients, and *M hominis* was found in four Two strains isolated from two patients could not be identified The strain recovered from the diphasic medium was a *M salivarium*

The occurrence of these mycoplasmas in the throat was correlated neither to diagnosis nor to the presence of any of the antibodies measured They were mainly found in adults as shown in Table 5 Both *M orale* type 1 and *M salivarium* were isolated from one of the patients In another patient the first throat swab yielded both *M pneumoniae* and *M salivarium*

Bacteria

After inoculation on mycoplasma media growth of bacteria or their L phase variants occurred from 30 of the throat swabs In eight of these cases the diphasic medium was used alone

Tracheal aspirations from 186 of the patients were routinely examined for bacteria This was performed in 139 adults and in 47 children and mainly in patients with pneumonia (127 cases) *Diplococcus pneumoniae* was dominant in this group None of these results could be correlated to isolation of mycoplasmas or bacteria and their L-phase variants by cultivation on mycoplasma media *Haemophilus influenzae* was cultivated from two patients with *M pneumoniae* illness

From two other patients with this diagnosis, and from eight patients with a positive *Influenza A* CF test, *Diplococcus pneumoniae* was cultivated

DISCUSSION

Among the 314 hospitalized patients examined during a two year period there were 246 patients with acute respiratory tract infection (162 with and 84 without pneumonia) and 68 control patients Evidence of *M pneumoniae* infection according to the criteria employed here was obtained in 12 all of whom belonged to the group with pneumonia (Table 4) Ten of these patients were hospitalized in the second one year period (March 1967 to March 1968) and nine accounted for an increase in incidence during the last five months The low number of *M pneumonia* infections found in the first period (March 1966 to March 1967) is probably not due to the negative isolation attempts in the first 85 patients where the diphasic medium was used alone since antibodies to *M pneumoniae* were found in only one of these patients This patient had no respiratory illness and the IHA and CA tests were negative in the only serum sample available, the positive FAT presumably reflected the persistence of antibodies raised during an earlier infection

During the whole study, a discrepancy between results of the FAT and IHA tests was noted in three patients this may be due to an individual variation within the immunoglobulin classes of the antibodies measured in either test (3)

A combination of the present and previous studies (21 22) gives the impression that *M pneumoniae* infections were more common in this country in the years 1962 to 1964 than in 1965 1966 and the first half of 1967 The shift in incidence during this study from low (March 1966 to March 1967) to high (March 1967 to March 1968) was substantiated by the results of serological investigations of patients with a positive CA test, they were randomly selected from the whole country during and beyond the same periods From December 1965 to January 1967 only 3 out of 100 patients had a positive FAT for antibodies to *M pneumoniae* while this test was positive in 32 per cent of 171 patients from the period December 1967 to October 1968 More than 80 per cent of all these patients suffered from acute respiratory illness (22)

It should be noted that the high incidence demonstrated in the last five months of the present study seemed to continue for at least the following six months, as mentioned above (22)

To these findings may be added the results of FAT carried out on sera from 174 patients hospitalized with acute respiratory illness in Copenhagen in 1957 and 1958 The percentage of patients with a positive anti *M pneumoniae* test was 25, indicating a high incidence of the illness at that time (22)

These data indicate that there have been appreciable fluctuations in the incidence of *M pneumonia* infections during one to two-year periods They are supported by reports of similar fluctuations in various areas where incidences have ranged from 1 to over 30 per cent of patients with pneumonia (4 6 14 18, 23 31) In other studies the incidence of *M pneumoniae* was found to be relatively stable for several years (2 11)

The number of patients with *M pneumoniae* infection in the present study increased during the last five months The small number of cases does not allow any estimate of a possible seasonal variation However when the results of this study are combined [illegible] those from serological studies of [illegible] materials (22) no seasonal variation [illegible] spect to the percentage of positive [illegible] *pneumoniae* test could be demon[illegible]d Lack of seasonal variations in the [illegible]ld has also been observed elsewhere (15 [illegible]ted

M pneumoniae infection was [illegible] art 84 patients with respiratory illness [illegible] al pneumonia diagnosed as lower respi[illegible] tract illness in 39 and upper respiratory [illegible]eem illness in 45 cases This [illegible] refle[illegible] pre relatively low overall in[illegible] of [illegible] other tion in other studies w[illegible] high [illegible]

respiratory illness due to *M pneumoniae* was also found in patients without pneumonia (11, 14, 2)

A positive CA reaction was found in 20 per cent of the present material in 26 per cent of patients with pneumonia, and in 12 of the 14 patients with a positive test for specific antibodies As *Biberfeld et al* (2) point out these incidences vary in different studies probably due to technical differences or to differences in the interval between the onset of the illness and the collection of specimens Another explanation of variations in the incidence of positive CA reactions in different studies of primary atypical pneumonia (p a p) is that other agents than *M pneumoniae* presumably give rise to production of CA, e g *Influen a* virus or *Adenovirus* (24 16, 22) Antibodies to these agents were in the present study found to be significantly correlated to the presence of a positive CA test Fluctuations in the prevalence of pneumonias due to *Influen a* or *Adenovirus* may therefore influence the incidence and significance of CA activity in studies of such materials

The frequent occurrence of a positive CA test in patients with *M pneumoniae* infection, however makes this test a useful diagnostic supplement It indicates recent or actual infection when found together with a positive FAT or IHA test for specific antibodies because cold agglutinins usually disappear earlier than the latter (16 22) For this reason a tive CA test was demanded in addition positive test for specific antibodies as one the criteria for the diagnosis of illness due *M pneumoniae* (see also 2)

The other criterium i e isolation of the agent could be criticized for not always demonstrating actual infection since *M pneumoniae* can be isolated from the throat up to 45 days after onset of illness (23, 4 20) However numerous observations favour the view that an isolation of *M pneumoniae* in the majority of natural infections indicates an actual or recent illness caused by this agent (24 4 1 6 11 23 26 2) The studies cited are also consistent with our findings that children and young adults most often contract the disease As shown previously (20), *M pneumoniae* could be isolated after the patient had been treated with tetracycline

The incorporation of methylene blue (MB) in media for the isolation and cultivation of *M pneumoniae* was introduced by *Kraybill & Crawford* (17), they found that this species tolerated 0 1 per cent MB in broth They used standard mycoplasma agar with 0 002 per cent MB for direct inoculation of throat swabs, this concentration inhibited the other known species of human mycoplasmas The results were roughly equal to those obtained by inoculation onto agar without MB The use of a diphasic medium with 0 001 per cent MB for isolation studies was recommended by *Chanock* (5) as later described by him and his co workers (6)

No reason for the observed inferiority of this medium compared with conventional mycoplasma agar plates can be given Experiments showed that even small inocula of the strains Mac and FH of *M pneumoniae* replicated in the medium a concentration of 0 01 per cent MB however was found to inhibit growth in broth of the Mac strain Different batches of MB are perhaps responsible for the discrepancy between results in this study and those of *Kraybill et al* (17) Presumably first passage isolates of *M pneumoniae* are less resistant to MB than the multiply passaged Mac and FH strains

Another reason for supplementing the MB diphasic medium with agar without MB was that growth of bacteria in the diphasic medium interfered with the detection of a potential growth of mycoplasma Colonies of bacteria or their L phase on mycoplasma agar medium never seriously interfered with the detection and isolation of mycoplasma

The isolation of 40 strains of mycoplasma other than *M pneumoniae* represents a minimum as the inoculum of the throat swab was not cultured under anaerobic conditions such conditions are preferable for the growth of *M salivarium M orale* types 1 and 2 and *M fermentans* (12 29 7) *M orale* type 2 and *M fermentans* were looked for but not found The higher isolation rate

for *M salivarium* than for *M orale* type 1 and *M hominis* is in accordance with the findings of *Organick et al* (26) and *Del Giudice et al* (9) In other reports *M salivarium* and *M orale* type 1 (= *M pharyngis*) have been detected with the same frequency (10 13, 26) The present data indicate that these mycoplasmas constitute a part of the normal pharyngeal flora in patients whether they suffer from respiratory illness or not As also shown by *Hendley et al* (13), the carrier rate increases with increasing age

The cultivation from tracheal aspirates of *Haemophilus influenzae* and *Diplococcus pneumoniae* from patients with p a p due either to *M pneumoniae* or possibly to *Influenza* virus may indicate that the patients had a secondary infection (14) However uncertain the aetiological significance of these bacteria may be, the findings show that their recovery by tracheal aspiration from patients with pneumonia does not exclude a mycoplasmal or viral aetiology (see also 23, 28)

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STUDIES OF BACTERIAL INTERFERENCE IN EXPERIMENTALLY PRODUCED BURNS IN GUINEA PIGS

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Thermal full thickness burns involving 2 per cent of the body surface area were produced in guinea pigs DNase and coagulase-negative staphylococci cultured from the animals normal bacterial flora were used as interference strain They were applied to the burns of the test guinea pigs As controls guinea pigs were used which were burned in the same way but not inoculated with the interference strain An easily identifiable strain of *Staphylococcus aureus* was subsequently sprayed on the burns in some of the series In the burns which were not sprayed with this test strain, records were kept only of spontaneously occurring *Staphylococcus aureus* In all the series the animals in the test groups showed significantly less growth of *Staphylococcus aureus* both with respect to the percentage of positive animals and to the colony counts from these animals *Staphylococcus aureus* positive guinea pigs thus were found in only 27 per cent in the test groups but in 73 per cent of the controls From the number of colonies per culture plate growth scores' were calculated from 0 to 4 according to an arbitrary scale The mean growth scores per animal of *Staphylococcus aureus* within the first two weeks after burning was 3 1 in the test guinea pigs and 8 8 in the controls. Possible practical consequenses of the observations made in the experiments are discussed, as for instance the possibility of bacteria belonging to the indigenous flora or other bacteria of lower pathogenicity to act as interference strains against hospital strains of pathogenic staphylococci

The experiments presented in this report were initially prompted by two observations made in bacteriologic studies in the Burns Unit of Karolinska Sjukhuset. Fresh, virtually bacteria free burns in hospitalized patients were found to be rapidly colonized by the pathogenic strains prevalent in the unit (*Burke et al 1960*) The second observation was made when erythromycin was given to two patients whose burns were complicated by staphylococcal septicemia (*Wickman 1957*)

Received 6 vi 69

During and after this treatment erythromycin resistant strains of staphylococci appeared throughout the unit and could be cultured from the burns of all the newly admitted patients But the resistant strains could not be demonstrated in patients admitted before the erythromycin treatment was started In all these patients the burns were already heavily infected with erythromycin sensitive staphylococcal strains It thus seemed that the first-established bacteria presented a hindrance to colonization by other bacteria in the environment

In the following study experimental burns were produced in guinea pigs and the intention was to explore the possibility of introducing into these wounds at an early stage bacteria innocuous to the host (*Staphylococcus epidermidis*) and, by maintaining their growth, to counteract invasion by microorganisms of greater pathogenicity (*Staphylococcus aureus*)

MATERIAL AND METHODS

Experimental animals Male albino guinea pigs weighing about 250 grams were used Their body surface was estimated according to the *Lee* (1929) formula *Liedberg* (1960) calculated a mean value for the constant used in the formula and his value was accepted as suitable for measuring burns in sufficiently large to affect the animals general health but not so small as to admit too rapid healing All guinea pigs came from the same breeder and had been kept on the same antibiotic free food The animals in one experimental series were all delivered at the same time and were of the same age During the experiments they were kept in single cages each with separate ventilation They were all kept on the same food supply to which no antibiotics had been added

Burning technique At least two and at most twenty four hours before burning the skin was disinfected and shaved over an area of about 16 cm on one flank The cautery consisted of a copper cylinder (basal contact surface 1 8 cm diameter 1 5 cm) connected to a soldering iron With the aid of a transformer the voltage in the cylinder was regulated and its temperature thus maintained at an appropriate level The cautery was heated to 375 C The prepared area of the skin was tightened with an aluminium ring with an inner diameter of 3 cm The cautery was rotated twice on this circular skin area This produced a full thickness burn measuring about 7 cm and comprising approximately 2 per cent of the total body surface area as calculated according to the mentioned formula The dry necrotic crusts that formed on the burns could readily be removed after seven days leaving a moist, sometimes slightly bleeding surface

This procedure was used in all the reported experiments except in the two preliminary series 19 and 24 In the 36 guinea pigs in these series the spontaneous shedding of the necrosis or the day when it could be removed with the utmost ease was awaited This day occurred at a mean interval of 8 7 (± 0 5) days after burning

Interference strains It was essential that the strains used as interference bacteria should be virtually non pathogenic to the host and capable of satisfactory proliferation in the burns Easy identification of the strains in cultures was also desirable

Optimum fulfilment of the first prerequisite seemed to be possible by use of one or more of the bacteria normally carried by the guinea pigs From oral swabs coagulase negative and DNase negative staphylococci* with a high natural resistance to tetracycline (M I C 50–100 μg per ml according to the method described by *Ericsson et al* 1954) were isolated and used in a preliminary series of experiments

Three such strains were tested – 18/49 and 18/511 (white pigment) and 12/60 (orange pigment) The three strains were tested with respect to their influence on the time and course of healing of the burns Three groups each of six guinea pigs were used for each of the tested strains All the animals were inoculated with the method described on p 3 but at different intervals and at different times after burning in the three pairs of animals in each group (Table 1) In six control guinea pigs no interference strains were applied to the burns

Strain 12/60 had the best persistance in the wounds as inferred from daily cultures after the last application of the strain This strain was therefore selected for use as interference strain in the definitive experiments According to *Baird Parker* (1965) this strain was to be regarded as a *Staphylococcus epidermidis* type 2

Pathogenic strains (deliberately applied or spontaneously appearing) In the earlier studies of bacteria in burns in hospitalized patients (*Birke et al* 1960) certain species were found to be the commonest causes of complications such as delayed healing rejection of grafts and sometimes bacteremia and septicemia Of these species β hemolytic streptococci and gram negative rods were very difficult to get to persist in the burns and for this and other reasons discussed later on a strain of *Staphylococcus aureus* was chosen for the definitive experiments The chosen strain called D 125 was coagulase positive also with guinea pig plasma and in contrast to most of the spontaneously appearing staphylococcal strains in guinea pigs it belonged to a definite phage type viz 29/81/KS6/6/7/42E/47 + I III (*Blair & Williams* 1961)

In order to further facilitate recognition of strain D 125 in cultures it was passed through liquid medium containing increasing amounts of erythromycin until a minimum inhibitory con

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* The coagulase producing capacity of these and other staphylococci was tested on human and/or guinea pig plasma at 37 C and their capacity of splitting desoxyribonucleic acid on DNA test medium (B B L) with 0 2 per cent DNA

TABLE 1 *Survey of Experiments*

Series number	Number of guinea pigs test*	controls†	Interference bacteria	Test strain staphylococci	First culture (days after burning)
19	18 (3 series of 6)	6	12/60 2 guinea pigs daily until crust removal; 2 guinea pigs daily until healing; 2 guinea pigs once after crust removal	None applied	3–10 (immediately after crust removal)
			18/49 ditto		
			18/511 ditto		
24	6	6	12/60 daily until crust removal	D 125 1 6 and 13 days after burning	8–17 (immediately after crust removal)
25	12	12	12/60 Immediately after burning; immediately after crust removal	D 125 2 days after burning; 2 days after crust removal	3
27	11§	12	12/60 as in series 25 + 12 days after burning	D 125 as in series 25 + 14 days after burning	5
28	11§	12	12/60 as in series 25	D 125 as in series 25	3
31	12	12	12/60 as in series 25	None applied	5
32	12	12	12/60 as in series 25	None applied	5

* Inoculated with interference bacteria
† Not inoculated with interference bacteria
§ One animal dead of intercurrent disease

centration of 50–100 µg per ml was reached The coagulase test on guinea pig plasma and the DNase test were still positive with the erythromycin resistant staphylococci Even with scanty growth the colonies of D 125 were easily recognizable because of their hemolysis and pigmentation This erythromycin resistant strain was also tested for virulence in guinea pigs by intravenous injection The animals died within one week The post mortem revealed pyemia and multiple abscesses with erythromycin resistant staphylococci in brain and kidneys

Application of the strains The *interference strain* was applied to the burns with the aid of a sterile cotton wool swab dipped into the dense growth of a blood agar culture The swab was rolled over the burned surface which was thus massively inoculated with the bacteria

The *test strain* D 125 was applied to the burned surface in an aerosol which was prepared of material from ten colonies on a blood agar plate suspended in 1 ml broth The suspension was diluted 1 100 and pipetted into the glass reservoir of a Hecto (ACO) atomizer an instrument designed for accurate dosage in aerosol of an adrenalin preparation in asthma From a distance of 3–5 cm the burned surface was sprayed with the bacterial suspension thrice on each occasion of treatment Immediately afterwards a blood agar plate was sprayed from the same distance and then incubated overnight after which the colonies were counted In this manner the number of bacteria reaching the burned surface could be estimated on each occasion In 14 sprayings performed on series 24–30 the mean number of colonies per 7 cm was 26 (±2 4) As the growth was always denser in the centre of the plate corresponding to the wound surface 30–40 should be a more correct estimation of the number of colony forming units

In some series of guinea pigs no test strain was applied (Table 1) Instead the spontaneous occurrence of staphylococci in the burns was studied

Culture technique Material from the burn area was collected with moistened sterile cotton wool

In the following study experimental burns were produced in guinea pigs and the intention was to explore the possibility of introducing into these wounds at an early stage bacteria innocuous to the host (*Staphylococcus epidermidis*) and, by maintaining their growth, to counteract invasion by microorganisms of greater pathogenicity (*Staphylococcus aureus*)

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RESULTS

Healing time No difference of healing time or of inflammatory reactions in the form of fissure formation erythema or suppuration was observed in the preliminary series 19 between the guinea pigs inoculated with the interference strains and those burned in the same way and at the same time but not so inoculated In one of the test guinea pigs there was delayed healing due to a deep fissure but this fissure arose at a time when the interference strain could no longer be cultured from the burned surface Excluding this animal and another which died of intercurrent disease before the burn healed the mean healing time in the 16 guinea pigs inoculated with the interference strains was 22 8 (± 0 6 days The mean healing time in the 6 controls was 22 6 (± 0 7) days

There was no significant difference in healing time between *Staph aureus* positive and negative guinea pigs Only in series 28 there was a tendency to longer healing time for the D 125 positive animals but the negative animals were too few to admit any definite conclusions

Bacterial Interference

Preliminary experimental series (series 19) This series was intented for testing the properties of different interference strains No staphylococcal test strain was applied However spontaneous colonization with *Staph aureus* occured to a degree which permitted comparisons with respect to the competitive effect of the different interference strains The results are presented in Fig 1 where the percentage of *Staph aureus* positive cultures is compared with the growth scores of interference bacteria The interference strains 12/60 and 18/49 were about equally effective as competitors against staphylococcal growth Of the cultures showing growth scores 3–4 of the interference strain only one of type 12/60 and none of type 18/49 contained *Staph aureus* Of these two strains 12/60 had the best persistance in the wounds as described in p 2

Definitive experimental series (24, 25 27, 28 31 and 32) The experimental procedure in these series is surveyed in Table 1

The occurrence of animals with more than scanty growth of interference strain 12/60 in the treated groups compared with tetracycline resistant staphylococci of the same type spontaneously occurring in the control groups is shown in Table 2 The number of animals showing such spontaneous growth was very small especially during the first week of observation In the test group, on the other hand, growth of 12/60 appeared in almost 100 per cent of the animals during the first two weeks but the number was gradually reduced to about half as many during the third and last observation week Therefore in calculating the number of *Staph aureus* positive animals in the different groups only the first two weeks are taken into consideration

The frequency of *Staphylococcus aureus* positive guinea pigs in treated and non treated groups is shown in Fig 2 As can be seen from this figure there was a difference with more positive animals in the non treated group at all the different sampling occasions Only at the last occasion 18–19 days after burning the difference was not significant (Table 3) At this time the burns were already healed or almost healed At all other times the difference was highly significant or significant the greatest difference occurring nine days after burning (two days after the removal of the crust) Of the 13 animals in the control group which were positive at the first sampling occasion (3–5 days after burning) most of them (11 animals) were found in series 31 and 32 where no D 125 was sprayed and thus only spontaneously occurring *Staph aureus* were registrated

The growth scores per animal of *Staph aureus* at different times after burning in series 25 27 28 31 and 32 are collected in Table 4 The mean scores per animal of the interference strain in the treated groups on each of the seven sampling oc asions are presented in F g 3 In the first tests made three to five days after burning the control

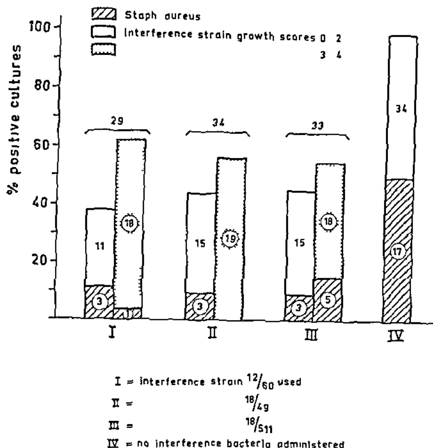


Fig 1 Series 19 Frequency of cultures showing *Staph aureus* in relation to treatment with interference bacteria and growth scores of interference strains The figures on the columns denote number of cultures

TABLE [illegible] *Interference Strain 12/60 (or Equivalent) in Treated and Non treated Guinea Pigs at Different Times after Burning*

Series 25 27 28 31 32

Days after burning	12/60 treated animals: Total number of animals	Interference strain growth scores 3–4*: Number	Per cent	No interference treatment: Total number of animals	Interference strain growth scores 3–4*: Number	Per cent
3 5	58	58	100	60	1	1 7
7	58	54	93	60	1	1 7
9	58	58	100	60	11	18
11–12	58	57	98	60	9	15
14	58	48	83	60	5	8
16	57	41	7[illegible]	60	4	7
18–19	40	20	50	45	5	11

* Scale of scores 0–4

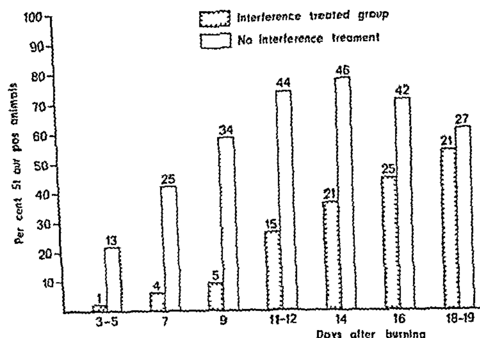


Fig 2 Series 24 25 27 28 31 32 Frequency of animals showing *Staph aureus* in interference treated and non treated groups at different times after burning The figures above the columns denote number of positive animals

TABLE 3 *Number of Staph Aureus Positive Guinea Pigs in Interference (12/60) Treated (A) and Non Treated (B) Groups at Different Times after Burning*

Series 25 27 28 31 32

Days after burning	Total number of animals A	Total number of animals B	Total number of pos animals A	Total number of pos animals B	Per cent pos animals A	Per cent pos animals B	χ	Significance of difference
3–5	58	60	1	13	2	22	11 2	***
7	58	60	4	25	7	42	19 2	***
9	58	60	5	34	9	57	30 8	***
11–12	58	60	15	44	26	73	26 6	***
14	58	60	21	46	36	77	19 7	**
16	57	60	25	42	44	70	8 2	
18–19	40	45	21	27	53	60	0 5	–

guinea pigs showed relatively poor growth of *Staph aureus* (mean scores per animal 0 9) but as the growth scores were extremely low in the animals of the test groups (mean scores per animal 0 09) the difference was highly significant In the next cultures made immediately after removal of the crust the difference per animal was not significant The scores both for the test animals and for the controls remained low (means per animal 0 6 and 1 1) Th result could seem to be in disproportion to the highly significant difference between treated and non treated groups on day 7 as regards the number of positive animals (cp Tables 3 and 4) This seems to be dependent on the occurrence in the treated groups in series 31 and 32 of animals with relatively high but transitional growth scores (On day 7 growth scores 32 4 positive guinea pigs on day 9 growth

TABLE 4 *Mean Growth Scores* per Guinea Pig of* Staph aureus *(Test Strain Plus Spontaneously Occurring) at Different Times after Burning*
Series 25 27 28 31 32

Time after burning (days)		After interference strain treatment	No interference strain treatment	Significance of difference
3–5	Number of animals	58	60	***
	Mean growth scores per animal	0 89	0 9	
7	Number of animals	58	60	–
	Mean growth scores per animal	0 6	1 1	
9	Number of animals	58	60	**
	Mean growth scores per animal	0 3	2 1	
11–12	Number of animals	58	60	***
	Mean growth scores per animal	0 9	2 5	
14	Number of animals	58	60	***
	Mean growth scores per animal	1 4	2 7	
16	Number of animals	57	60	**
	Mean growth scores per animal	1 7	2 7	
18–19	Number of animals	40	45	–
	Mean growth scores per animal	1 9	2 2	

* Scale of scores = 1–4

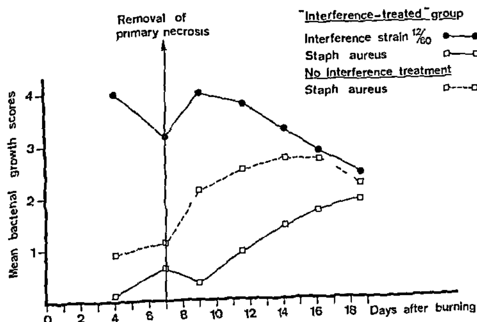


Fig 3 Series 25 27 28 31 32 Bacterial growth scores at different times after burning
Total number of animals day 3–14 = 118 day 6 = 117 day 18–20 = 75

TABLE 5 *Frequency (per cent) of* Staph aureus *positive Guinea Pigs after Treatment with Interference Strain 12/60**

Series 24 25 27 28 31 32

	Number of animals	After interference treatment	No interference treatment
Staph aur pos	65	17 = 27 %	48 = 73 %
Staph aur neg	65	47 = 73 %	18 = 27 %
Total	130	64 = 100 %	66 = 100 %

χ = 26 7 *Significance of difference* $p < 0\,1\,\%$ = **

* Samples taken within two weeks after burning

TABLE 6 *Growth Scores of* Staphylococcus aureus *in Guinea Pigs Treated with Interference Strain 12/60†*

		Number of Series A	Number of Series B	Number of Animals A	Number of Animals B	Mean growth Scores per series A	Mean growth Scores per series B	Difference	Mean difference per animal	Significance of difference
Staph aureus	D 125	4	4	40§	47	6 1	12 4	6 3	1 6 ± 0 9	–
	Other	6	6	64§	66	12 7	41 4	28 7	4 8 ± 0 5	*
	All	6	6	64§	66	18 7	53 0	34 3	5 7 ± 0 3	* *

Mean growth scores per animal A (after interference treatment) = 3 1
B (no interference treatment) = 8 8

Scale of scores = 0–4

† Samples taken within two weeks after burning

§ Two animals dead of intercurrent disease

scores 9 3 positive guinea pigs) This phenomenon will be discussed later on The greatest difference was found on day 9 when the mean scores were 0 3 for the test animals and 2 1 for the controls In the following tests made at intervals of one to three days the difference was highly significant within the first two weeks after burning but it gradually diminished and in the final tests 18 to 19 days after burning it was no longer statistically significant

Table 5 deals with all the six definitive series and shows the total frequency of *Staph aureus* positive animals within the first two weeks after burning The difference between the interference treated and non treated groups was highly significant with 27 per cent positive animals in the test groups and exactly the same percentage of negatives in the control groups

In Table 6 are registered the mean *Staph aureus* growth scores separately for strain D 125 and other *Staph aureus* As in Table 5 they are collected from all the six definite series and within the first two weeks after burning The difference in growth scores per animal between the guinea pigs treated with interference strain 12/60 and the untreated animals was highly significant as regards the spontaneously occurring *Staph aureus* and if all *Staph aureus* are included irrespective of origin and type With test strain D 125 the difference was not significant In fact there was only one series (series 28) where most of the burns in the untreated group of animals became massively colonized with test strain D 125 In this series as in all but one of the others there was no animal positive for both D 125 and spontaneously occurring *Staph aureus* Mean staphylococcal growth scores per animal was 3 1 for treated and 8 8 for untreated animals

TABLE 7 *Series 25 and 28 Guinea Pigs Positive (+) or Negative (—) for Different Types of* Staph aureus *in Interference Treated and Non Treated Groups**

Guinea pig number		Series 25				Series 28			
		A		B		A		B	
A	B	D 125	Other	D 125	Other	D 125	Other	D 125	Other
1	13	—	—	—	+	+	—	+	—
2	14	—	—	—	+	—	—	+	—
3	15	—	—	—	+	§	§	+	—
4	16	—	—	—	+	—	—	+	—
5	17	+	—	—	—	—	—	+	—
6	18	—	—	+	—	—	—	+	—
7	19	—	—	—	—	—	—	+	—
8	20	+	—	—	+	+	—	—	+
9	21	+	—	+	—	+	—	—	+
10	22	—	—	—	+	—	—	—	—
11	23	—	—	—	—	—	—	—	+
12	24	—	—	+	—	—	—	—	+
Total no of *Staph aur* pos animals		3+		9+		3+		11+	

A = after interference treatment
B = no interference treatment

* Samples taken within two weeks after burning
§ Guinea pig dead of intercurrent disease

TABLE 8 *Interference Strain 12/60 (or Equivalent) Growth Scores** *in* Staph aureus *Positive and Negative Guinea Pigs*†

Series number	Number of animals		12/60 growth scores		Mean 12/60 growth scores per animal		Mean difference per animal of 12/60 growth scores
	Staph aur positive	Staph aur negative	Staph aur positive	Staph aur negative	Staph aur positive	Staph aur negative	
24	5	7	110	176	22 0	25 1	3 1
25	11	13	62	205	5 6	15 8	10 2
27	12	11	39	180	3 3	16 4	13 1
28	14	9	101	155	7 2	17 2	10 0
31	11	13	103	199	9 4	15 3	5 9
32	12	12	101	162	8 4	13 5	5 1
Total	65	65	516	1077	55 9	103 3	7 9±1 6

Significance of difference $p < 1\%$ — **
* Scale of scores 0–4
† Samples taken within 2 weeks after burning

Series 25 and 28 can be referred to as typical examples of tendency to interference between different strains of *Staph aureus* (Table 7) Series 25 had no animals positive for spontaneous *Staph aureus* in the test group but six such animals in the control group Strain D 125 occurred in as many animals as the spontaneous staphylococci, but they were equally distributed in both the groups with only three in each

In series 28 the results in the non treated animals were in striking contrast to those in

series 25 Thus there were seven animals positive for strain D 125 in this group and only four were positive for other *Staph aureus* In the test group on the contrary, the results were very much alike those of series 25 with three animals positive for D 125 and the rest negative for both types of *Staph aureus*

In Table 8 the animals in the six series are divided into two groups one including the *Staph aureus* positive guinea pigs and the other the negative ones Within those two groups are registered the total growth scores of each animal for the interference strain 12/60 estimated in the same way as *Staph aureus* growth scores in the preceding tables The mean growth scores per animal of strain 12/60 are compared The result of these calculations is that there was a statistically significant difference of 12/60 growth scores between the two groups The mean difference of growth scores between the 65 animals in each group was 7 9 (± 0 2)

DISCUSSION AND CONCLUSIONS

Observations made in connection with a bacteriologic and hygienic investigation in the Burns Unit of Karolinska Sjukhuset provided the impetus for the experiments here described When patients were admitted with extensive burns these were rapidly colonized by the bacteria prevalent in the department The bacteria first established in the burns seemed as a rule to prevent or diminish infection with other bacteria in the environment This appeared to be especially true of closely related bacteria for example various types of strains of staphylococci

Similar observations have been made in the U S A in connection with nosocomial staphylococcal infections in neonates Attempts have been made to exploit the phenomenon prophylactically (*Shinefield et al 1963*) using as the interference micro organisms a coagulase positive phage typed staphylococcal strain called 502 A This strain was considerably less virulent than the staphylococci which had caused a number of severe nursery infections over a long period Strain 502 A was subsequently used as a blocking agent in a number of clinical studies in neonates (*Light et al 1965)*, in healthy adults (*Boris et al* 1964) and in a case of furunculosis (*Strauss et al* 1965)

The blocking agent used by these writers thus was a potentially pathogenic microorganism which however, had only occasionally been cultured from minor pyogenic lesions The first report in which strain 502 A could be associated with a more virulent infection was published in 1966 by *Drutz et al* The patient had furunculosis and was given 502 A intranasally During topical steroid treatment of the furunculosis 502 A was isolated from abscesses in pure cultures on two occasions

In my experiments on guinea pigs the interference strain could be regarded as wholly innocuous It was obtained from the animals' indigenous flora and tests of pathogenicity (DNase and coagulase tests) were negative In preliminary experiments burns were massively inoculated with the interference strains, but healing was not delayed and inflammatory reactions did not occur

In the definitive experiments half of the animals were treated with an interference strain which was applied to the burn immediately after its infliction and immediately after the necrotic crust had been removed or shed On these occasions the natural bacterial population presumably had fallen to a minimum level and thus the applied strain could be said to have invaded an unoccupied site

In some series all the burns were later sprayed with an aerosol of coagulase positive staphylococci In the other series only spontaneously occurring staphylococci were registered

In no series was colonization by *Staphylococcus aureus* wholly prevented The percentage of animals showing growth of *Staphylococcus aureus* however, was considerably less in the groups where the burns had been treated with interference strain than in the untreated groups When the total growth of

Staphylococcus aureus (applied plus spontaneously occurring) was considered, there was also an appreciable difference in the massiveness of growth which remained statistically significant except for the final tests 18 to 19 days after burning

On the seventh day after burning there was a discrepancy between the massiveness of growth (no difference in growth scores between the treated and untreated animals) and the number of *Staph aureus* positive animals (a highly significant difference between the two groups) Regarding the definition of a positive animal as a guinea pig with at least two consecutive positive cultures, this divergency could depend either on an occurrence in the *treated* series of *Staphylococcus aureus* with relatively *high* growth scores which had disappeared at the next sampling occasion or it could depend on samples from the *untreated* series of *low* growth scores followed by an increase of growth at the next sampling occasion Both these possibilities could theoretically be attributed to an interference by 12/60 in the treated guinea pigs or a lack of interference in the untreated animals At least in series 31 and 32 the growth scores were high in relation to the number of positive animals in the treated groups and therefore it could have been the first mentioned possibility that was relevant

The observation that the pathogenic micro organisms finally prevailed seems to imply that the interference bacteria did not exert a true antagonistic effect and did not confer immunity but that the phenomenon was merely one of competition between bacteria with approximately equal demands on the available nutrients If, however the interference colonization could be maintained at a high level throughout the experimental period the results presumably would be different This was indicated by the calculations in which the frequency of animals showing *Staphylococcus aureus* growth was correlated to the growth scores of interference bacteria

Priority of colonization important

Anthony & Wannamaker (1964), for instance, tested various strains of *Staphylococcus aureus* in experimentally produced burns in rabbits and found that the first applied strain, whether this was the most commonly used 502 A or some other prevented subsequent colonization by other strains of *Staphylococcus aureus*

Simon (1965) observed that a strain of *Staphylococcus aureus* which had been applied in the bacteria free noses of guinea pigs only very seldom was replaced by a superinfecting strain The original strain became resident in the noses, in some cases alone but in about half of the cases together with the new strain

In most of my experimental series the differences regarding the occurrence of *Staphylococcus aureus* were ascribable to a difference in spontaneous colonization between the test guinea pigs and the controls Only in one series did the aerosol administered test strain gain such a firm foothold as to cause a significant difference in this respect between the test animals and the controls In this same series the animals showed relatively poor growth of other staphylococci This competition between different types of *Staphylococcus aureus* was often striking In the whole material of 82 animals sprayed with strain D 125 only one of the 42 *Staphylococcus aureus* positive guinea pigs was positive both for the test strain and spontaneous strains within the first two weeks after burning

In this connection it is relevant that even the coagulase positive phage typable D 125 staphylococci whose virulence had been tested by intravenous injection in guinea pigs mostly had no influence on the duration or course of healing The only exception from this rule was one series where the burns had been made somewhat deeper than in the other series Even in this series however, the burns were small and produced no general effect on the animals

Another phenomenon that could perhaps be interpreted as a sign of interaction between the two types of *Staphylococcus aureus*

(D 125 and spontaneously occurring) was the concentration to the series 31 and 32 of *Staphylococcus aureus* positive guinea pigs at the first sampling occasion 3–5 days after burning In these two series no D 125 had been administered There is a possibility that the prolongation of the incubation period in the other three series could be attributed to an initial interference between the two types of *Staphylococcus aureus*

In the earlier mentioned bacteriologic studies on burns (*Birke et al* 1960) 40 per cent of the deaths were due primarily to septicemia *Staphylococcus aureus* was the bacterial species most commonly isolated in blood cultures from these patients Of 82 patients with *Staphylococcus aureus* in exudation from burns 46 per cent had elevated antistaphylolysin titres which indicates that the effect of these bacteria was not only a local one

Balch (1963) stated that the primary cause of bacteremia and septicemia in burned patients probably is the presence of a high bacteria/cell ratio in the wound Also *Moncrief* (1966) made the observation in burned patients that survival it not dependent upon sterilization of the burn wound but upon limiting the magnitude of the infection so that the balance between host and infection favours the host It follows therefore that the local growth of bacteria must be kept to a minimum in order to prevent generalized invasion of the blood stream

The results of this investigation seem to underline the importance of seriously considering the risks of creating by antibiotic prophylaxis or otherwise a reverse result of the bacterial competition In these cases the originally present antibiotic sensitive and often relatively non pathogenic strains which in the referred experiments were represented by the interference strain are eliminated leaving the field free for the more resistant and often more virulent bacteria which tend to flourish in hospitals Of these β hemolytic streptococci after the introduction of penicillinase resistant penicillins are no longer a real threat, not even in the presence of penicillinase producing staphylococci Pseudomonas and other gram negative rods are more difficult to handle but in contrast to the situation in most other places infections with these bacteria do not seem to have increased in frequency or seriousness in the Stockholm material Furthermore the colonization of the burn wounds with Pseudomonas does not seem possible to prevent with any sort of antibiotic prophylaxis (*Wickman* 1970)

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A NEW EPIDEMIC PHAGE TYPE OF *STAPHYLOCOCCUS AUREUS*

3 *Epidemic Spread of Phages among Danish Hospital Staphylococci*

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In *Staphylococcus aureus* strains of type 83A/6557 and in type 6557 and other group III strains insusceptible to phage 83A two kinds of temperate phages were frequently found belonging to the serological group A (α phages) and B (β phages), respectively The two phages have been characterized by conventional criteria including plaque morphology serological grouping host range and ultrastructure To meet with the need for more specific identification the following characteristics were included lysogenic conversion—*in casu* the ability to convert a positive Tween 80 reaction to a negative one—ability to block susceptibility to certain typing phages and kinetics of inactivation with specific anti phage sera These tests were found valuable and may deserve wider application as criteria in phage identification It is made probable that the β phage has been responsible for the evolution from type 83A/6557 to type 6557 due to its ability to block susceptibility to phage 83A Suggestive evidence is presented for an actual epidemic spread of this particular phage within the existing population of susceptible hospital staphylococci contributing materially to the recently observed change in prevalent epidemic types

In previous papers it was suggested that a spread of certain phages might have occurred among hospital staphylococci of the type complex III, 83A 6557 (*Bulow* 1968b, c) It was assumed that the phages by lysogenization blocked susceptibility to phage 83A and some other typing phages thereby altering the phage types A simultaneous lysogenic conversion from a positive to a negative Tween 80 reaction was often noticed

The evidence of such a spread of epidemic phages in Danish hospitals during the years 1962–1965 is here presented in more detail than was possible in a preliminary brief congress communication (*Bulow* 1967)

Received 17 vi 69

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MATERIAL

The strains examined for phage production were taken at random from the two materials of hospital staphylococci described earlier (*Bulow* 1968b)

Test strain 11136 used for the detection of phages in strains of the III 83A 6557 complex was selected from a number of group III strains screened for this purpose By the chloroform method of *Kjems* (1955) it was found that the majority of strains of the types 83A/6557 and 6557 produced phages when plated on test strain 11136 The phage type of test strain 11136 was 31B/5?B/83A/6557 it gave a positive Tween 80 reaction

METHODS

Phage types antibiotic sensitivity and the ability to split Tween 80 (abbreviated TW) were determined as previously described (*Bulow* 1968a) Abbreviations used in antibiograms are given in the *legend of Table 1*

Phage production from staphylococci was demonstrated by the chloroform method (*Kjems* 1955) and by the living cross method (*Fisk* 1942)

The agar layer method *of Swanstrom & Adams* (1951) was used for the propagation of phages and in the study of plaque morphology where it had advantages superior to the simple surface agar tecnique Strain *11136* was used as propagating strain (PS) and as test strain (TS) *for the phages*

Lysogenization was carried out as described by *Rountree (1959)* The criteria for lysogeny were *phage production and immunity to the lysogenizing* phage

Serological grouping of the phages was performed as described by *Rountree* (1949) and *Rippon (1956)*

Anti phage rabbit sera were produced by subcutaneous inoculations of phage suspensions with 10^{10}–10^{11} plaque forming units per ml Inoculations were given every second day of 14 days *with doses increasing from* 0 25 to 1 0 ml and the rabbits were bled one week after the *last* inoculation For each of the two phages studied the serum with the highest velocity constant for in*activation of the homologous phage* was selected from three sera for each phage

Velocity constants (K) for phage inactivation by specific antiphage sera were determined from the equation

$$K = \frac{2\,3\,D}{t} \log_{10} (P_0/P_t) \text{ min}^{-1}$$

where D is the dilution of serum t is the time in minutes P_0 and P_t are phage counts *per ml* at the time zero and t *respectively* (*Adams* 1959)

The lytic spectra of the phages were determined according to the method of *Blair & Williams* (1961)

Electron microscopy was *performed* by *Karl Ho and Hougen* and *Aksel Birch Andersen* at the Department of Biophysics Statens Seruminstitut Copenhagen

Six ml of a dense phage *suspension in* broth (*about 10 ¹ particles per* ml) were centrifuged for 30 minutes at 65000 g in a Spinco Model L centrifuge The pellets obtained were thoroughly resuspended in 0 5 ml of a 1 per *cent ammonium* acetate pH 7 *Negative* staining (*Brenner & Horne* 1959) was carried out after mixing with equal volumes of a 2 per cent ammonium molybdate adjusted to pH 7 with *ammonium* hydroxide The mixture was placed on carbon reinforced Formvar coated grids using the loop method (*Murray* 1963 *Jepsen et al* 1968) Electron microscopy was performed on a modified Philips EM 100 B or a Philips EM 200 *electron microscope* Negatives were obtained at a primary magnification of about 9000 × on 35 mm Kodak Fine Grain Safety Positive Film and prints were made by *photographically enlarging as desired*

RESULTS

Isolation of Temperate Phages with Different Plaque Morphology from Strains of Type 83A/6557 and 6557

By the chloroform technique most strains of the types 83A/6557 and 6557 were shown to produce phages when plated on TS 11136 *In soft agar layers the phages produced two distinct kinds of plaques* here called α and β plaques shown in Fig 1

The α plaques are rather small about 0 75 mm in diamter but heterogeneous in size and transparency The β plaques are larger about 2 mm in diameter uniform in size and *transparent*

Other characteristics of the phages and their donor strains are given in Table 1 By choosing TW— donor strains it was possible to select lysogenized colonies from TW+ receptor strains, as these were converted to TW— ones The donor *strains had no known* epidemic *connection*

α *phages were found* in 55 per cent of type 83A/6557 TW— strains and the figure increases to 75 per cent when only strains resistant to PST are considered Only 3–4 per cent of the type 83A/6557 yielded β phages

In TW— strains of type 6557 α phages were rare (1–2 per cent) but β phages common They were however confined to strains with the antibiograms PSTENB PSTE or PST where they were found *with a frequency* of 82 per cent and *were never found in* strains with the *antibiograms* P PS PT or PSTEO

A total of 10 out of the 304 strains studied produced both α and β phages namely 6 strains of type 83A/6557 and 4 of type 6557 all of them resistant to PST

TABLE 1 *Characteristics of TW Negative Donor Strains of Phage Types 83A/6557 and 6557 Isolated during the Years 1963–1965 with Special Reference to the Content of Phages*

Phage type	Antibiogram	Number of strains	Small plaque phages (α)	Large plaque phages (β)
83A/6557	PST	121	91	6
	PS or PT	38	4	0
	sens or P	14	1	0
Total		173	96	6
6557	PST(C)ENB	178	0	146
	PSTEO	16	0	0
	PSTE	34	0	28
	PST	38	4	21
	PS or PT	26	4	0
	sens or P	12	1	0
Total		304	9	195

TW = Tween 80 reaction Sens = sensitive to all antibiotics
P = resistant to penicillin S = resistant to streptomycin T = resistant to tetracyclines (C) = resistant or sensitive to chloramphenicol E = resistant to erythromycin N = resistant to neomycin (and closely related compounds) B = resistant to bacitracin and O = resistant to oleandomycin
Strain 11136 described in the text has been used as test strain

TABLE 2 *Characteristics of TW Negative Donor Strains of Phage Group III with Special Reference to the Content of Phages*

Phage type	Antibiogram	Number of strains	Small plaque phages (α)	Large plaque phages (β)
III/83A/6557	PST	23	6	2
	PS PT P or sens	46	12	3
Total		69	18	5
III or III/6557	PST(C)ENB	56	0	45
	PSTEO	17	0	0
	PSTE	33	4	24
	PST	14	4	4
Total		120	8	73

III means reaction with one or more of the group III typing phages
For further explanation see Table 1

Isolation of Temperate Phages from Other Group III Strains

It was observed earlier (Bulow 1968b c) that resistance to E O N and B occurred in some group III strains insusceptible to phage 83A but not in those that included 83A in their phage typing patterns

For this reason the occurrence of temperate phages was studied in these two categories of strain i.e. group III strains insusceptible to phage 83A (here called III/6557 or III) and group III strains susceptible to phage 83A (here called III/83A/6557) Strain 11136 was again used ...

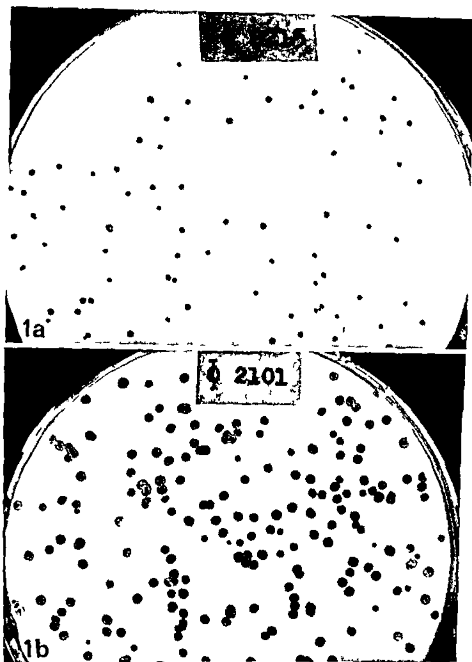


Fig 1 The plaque morphology of the α phages (1a) and the β phages (1b) using the agar layer technique (*Swanstrom & Adams* 1951) The agar plates have been incubated at 30 C for 24 hours

Within the first category 80 per cent of multiple resistant strains with the antibiogram PSTENB contained β phages but the oleandomycin resistant ones did not A few strains with a more limited resistance (PSTE PST) contained α phages

In the second category (III/83A/6557) 26 per cent of the strains contained α phages whereas β phages were rare

Thus it was evident that phages characterized as α and β according to their plaque morphology were common not only in strains

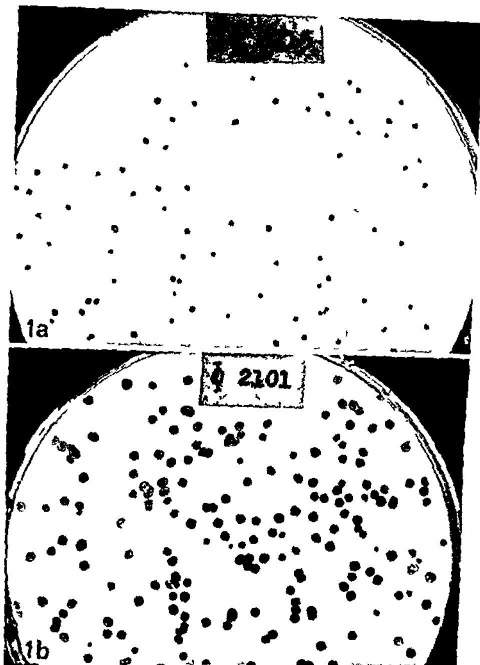


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In the second category (III/83A/6.57) 26 per cent of the strains contained α phages whereas β phages were rare

Thus it was evident that phages character ized as α and β according to their plaque morphology were common not only in strains

TABLE 3 *Influence of Lysogenization on the Susceptibility to the Typing Phages of Group III in Staphylococci with Various Typing Patterns*

Lysed by the typing phages			Lysogenizing phages	
Phage No	Serol group	No of strains	α	β
6	A	62	0	+*
7	A	60	0	0
42E	A	19	0	+*
47	A	74	0	+*
53	B	80	0	—*
54	A	64	—*	0
(81)	A	16	0	0
75	A	61	—	0
77	F	28	0	0
83A	B	50	0	—
6557	B	94	0	0
31B	B	21	—	—
42B	A	10	0	0
47C	A	15	—	—*
52B	B	24	0	—
73	A	11	0	—*
75A	A	26	—	0
83B	B	22	0	—
Serological group			A	B
Phages derived from strains of type			83A/6557	6557

10 α and 10 β phages have been used in the experiments All of the phages were able to convert the TW reaction from a positive to a negative one

0 = no change in typability
\+ = gain in sensitivity to typing phages
— = loss in sensitivity to typing phages
* = frequent (30–60 per cent) but not a constant change

TABLE 4 *Effect of Lysogenization of Strain 11136 with α and β Phages*

Strain	11136	11136 (α)	11136 (β)
TW reaction	+	—	—
Lysed by the typing phages	31B 52B 83A 6557	52B 83A 6557	6557

11136(α) = strain 11136 lysogenized with α phage
11136(β) = strain 11136 lysogenized with β phage

The TS 11136 (TW+ type 31B/52B/83A/6557) was easily lysogenized by each of the 10 α and 10 β phage isolates From each of the 20 converted cultures 10 colonies were subcultured and re examined for phage type and TW reaction

The results for cultures lysogenized by each of the two categories of phages were quite uniform as is seen from Table 4

A small number of phages (Tables 1 and 2) were atypical with regard to converting ability or ability to block susceptibility to typing phages The six β phages isolated from the 83A/6557 strains appearing in Table 1 the five β phages from type III/83A/6557 appearing in Table 2 and three out of the four β phages from type III or III/6557 lack either the TW converting property or the ability to change characteristically the phage type of TS 11136 by lysogenization

TABLE 5 *Host Range of α and β*

Phages isolated from type and (antibiogram)	Total number of phages	29	52	52A/79	80	2009	71
Type 6557 strains (PSTENB)	32	–	–	–	–	–	–
Group III/6557 strains (PSTENB)	27	–	–	–	–	–	–
Type 83A/6557 strains (PST)	28	–	–		–	–	–

(*) = inhibition
Strength of phage reaction given in brackets
5 = maximum titre on homologous propagating strain
4 = 10 ¹–10 of titre on the propagating strain

4 *Host Range*

The host range or lytic spectrum of 59 β and 28 α phages isolates was examined by means of the conventional set of 15 test strains (Table 5) Before the test was per formed all phages were propagated on strain 11136 in order to avoid host induced modifi cations (*Ralston* & *Kreuger* 1954, *Luria* 1953)

Table 5 shows that the β phages isolated generally had identical lytic spectra except that only 8 out of 32 β phages from typical ' type 6557 strains lysed PS 73 whereas all the 27 phage isolates from strains with the phage type III/6557 did so

The lytic spectra of the α phage isolates were uniform They differ distinctly from the pectra of β phages although it is noteworthy that the two categories of phages have many reactions in common

5 *Kinetics of Inactivation with Specific Anti phage Sera*

The velocity constant (K) for inactivation of the α phage (Φ 8215) with the selected homologous antiserum (s 1143) was as an average of five determinations Kα = 114 min ¹ If w re hat) = 0 05 the li mits will be Kα 16 min ¹

Determined in the same way and with the same probability limits of 95 per cent the K value for the selected anti β serum (s 1148) and the homologous β phage (Φ 2101) was Kβ = 5u ± 8 min ¹

In single experiments K values were de termined for the inactivation of six other α (group A) phages by the anti α serum and the anti β erum respectively The results are given in Table 6 as mean values of three experiments

The results suggest serological identity of the α phages from different type 83A/6557 strains and an α phage from a type III/83A/ 6557 strain An α phage originating from a group I strain (but—like the other phages—propagated on PS 11136) is erologically di stinctly different the velocity constant being less than one half of the values found above

Two α phages from older isolates (1957–1962) of type 6557 strains also differ from those isolated from type 83A/6557

No serological difference is demonstrated between β phage isolates from type 6557 and those from multiple resistant type III/ 6557 strains (i e insusceptible to phage 83A) mentioned in a previous paper (*Bulow* 1968b) All these β phages differ serologi cally from the two β (group B) phage iso lates from strains of the 52 52A 80 81 com plex (Table 6)

Phages Isolated from Various Strains

Propagating strains									Serol group
8719	42C	42E	47	53	54	73	75	77	
–	–	– (0)	32 (5)	32 (5)	32 (5)	8 (4–5)	31 (4)	32 (5)	B
–	–	– (0)	27 (5)	27 (5)	27 (5)	27 (4)	27 (4)	26 (5)	B
–	–	28 (3)	– (0)	28 (*)	28 (5)	28 (5)	28 (0–2)	– (0)	A

3 = 10^{-3}–10^{-4} of titre on the propagating strain
? = 10^{-5}–10^{-6} of titre on the propagating strain
1 = very weak lysis
– = no reaction

6 *Electron Microscopy of the Phages*

A clear connection between phage morphology and serological grouping has been described by *Seto et al* (1956) and *Rosenblum & Tyrone* (1964) and electron microscopic studies are not supposed to add important new information to the identification criteria mentioned above

Group A phages have elongated heads with rounded ends They are here about 95 mμ × 50 mμ in size, and the tails are relatively long about 300 mμ

Group B phages have round or polygonal heads with a size about 50 mμ × 50 mμ The tails are about 155 mμ

The results of the present investigation which included two α and three β phages are shown in Figs 2 3 and 4

TABLE 6 *The Velocity Constant (K) of Inactivation of Phages from Different Strains of Staphylococci by Specific Anti Phage Serum*

Designation of phage and donor strain	Serol group	Isolated from phage type	Antibiogram	K value (min⁻¹)
8?15*	A	83A/6557	PST	114
5944	A	83A/6557	PST	110
1458	A	83A/6557	PST	104
11269	A	III/83A/6557¹)	PST	106
327	A	52 5?A 80 81	P	45
240	A	6557 **	PST	54
312	A	6557 *	PST	5?
2101*	B	6557	PSTENB	55
14768	B	6557	PSTE	51
1703	B	6557	PSTCENB	50
5685	B	III/6557)	PSTE	5?
3051	B	III/6557³)	PSTENB	51
5681	B	III/6557⁴)	PSTE	54
688	B	5? 52A 80 81	P	3?
1478	B	5? 5?A 80 81	P	20

* Homologous
** Bacteraemia 1957–61
1) Complete phage type 6/47/53/77/83A/6557
2) Complete phage type /54/75/77/6557
3) Complete phage typ /75/77/6557
4) Complete phage t 54/75/77/6557

TABLE 5 *Host Range of α and β*

Phages isolated from type and (antibiogram)	Total number of phages	29	52	52A/79	80	?009	71
Type 6557 strains (PSTENB)	32	–	–	–	–	–	–
Group III/6557 strains (PSTENB)	27	–	–	–	–	–	–
Type 83A/6557 strains (PST)	28	–	–	–	–		–

(*) = inhibition
Strength of phage reaction given in brackets
5 = maximum titre on homologous propagating strain
4 = 10 ¹–10 of titre on the propagating strain

4 *Host Range*

The host range or lytic spectrum of 59 β and 28 α phages isolates was examined by means of the conventional set of 15 test strains (Table 5) Before the test was per formed all phages were propagated on strain 11136 in order to avoid host induced modifi cations (*Ralston & Kreuger 1954 Luria 1953*)

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In single experiments K values were de termined for the inactivation of six other α (group A) phages by the anti α serum and the anti β serum respectively The results are given in Table 6 as mean values of three experiments

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antibiotic resistance by transduction has been demonstrated and will be reported in a separate paper

The rapidly increasing number of multiple resistant strains of these types in Danish hospitals in the period 1962–1965 (*Bulow* 1969c) gives rise to two hypotheses 1) a simple spread of one or a few bacterial strains with epidemic properties of particular advantage to them in the present hospital milieu or 2) an epidemic spread of phages among the hitherto prevalent hospital flora of type 83A/6557 and other group III strains

If we accept the conclusion that the same β phage is found in the strains of type 6557 and type III/6557 it is a great argument in favour of the second hypothesis that this phage has spread simultaneously among endemic strains of type 83A/6557 and among the broad heterogeneous flora of other group III strains (here briefly designated III/83A/6557) in both cases accompanied by a change in antibiotic sensitivity These evolutionary processes may be summarized thus

1) 83A/6557 → 6557
2) III/83A/6557 → III/6557

Such virus epidemics among prevalent hospital staphylococci do not rule out the first hypothesis on the contrary it is evident that the evolution is followed up and reinforced by a local spread of staphylococci of the new types (*Bulow* 1968c)

From evidence at hand we can suggest the courses of other phage epidemics also One might imagine that waves of epidemic phages of different origins from time to time sweep over the staphylococcal flora in hospitals—maybe within limited geographical areas—giving rise to changes which render the staphylococci more fit for surviving the various influence in modern hospital surroundings

In Great Britain a phage dependent evolution from type 83A to a new type (now called 84/85) probably identical with the Danish type 6557 (*Bulow* 1968a) was described by *Jevons & Parker* (1964) However attempts to demonstrate the presence of identical phages in the new type were not successful The British type 83A strains were typically TW+ and erythromycin resistant (96 per cent and 63 per cent respectively (*Jevons et al* 1966)) whereas the Danish ones were TW— and erythromycin sensitive (81 per cent and 94 per cent respectively (*Rosendahl & Jessen 1964*)) The parent strains in the evolutionary process 83A/6557 → 6557 may thus have differed from one country to another Even if the descendants had a number of properties in common *e g* phage type and resistance to neomycin and bacitracin the further evolution from type 6557 (or more correctly 84/85/6557 to 84/85 6557 (only) 84 or 85) in Denmark characterized by being methicillin resistant but sensitive to erythromycin neomycin and bacitracin (*Jessen et al* 1969) may indicate a different pathway in the evolutionary processes Thus methicillin resistance has been a common phenomenon in Denmark during the last 3 years (about 30 per cent of isolates) whereas the British staphylococci are more seldom resistant to this antibiotic (*Parker* 1968 personal communication)

It is also suggestive that type 83A strains have never been widespread in North America and the rare occurrence of methicillin resistant staphylococci (*Barrett et al* 1968) may be explained as an outcome of a different basic hospital flora from that found in many European countries

The plentiful incidence of lysogenic staphylococci implies a constant presence of free phages able to lysogenize and probably convert other staphylococci in the environment The conversion may provide selective advantages for the receptor strains, *e g* immunity to related phages further more genetic material determining antibiotic resistance may be transferred Although only a very small proportion of free phages have transducing properties their effect may be considerable if a selective pressure due to antibiotics in the environment favours the new resistant variants

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SYNERGISTIC EFFECT IN VIRAL-BACTERIAL INFECTION

2 *Influence of Viral Infection on the Phagocytic Ability of Alveolar Macrophages*

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The influence of prior virus infection on the antibacterial activity of mouse alveolar macrophages against *Haemophilus influenzae* was investigated Parainfluenza 1 virus inhibited the phagocytic activity both after *in vivo* and after *in vitro* infection The capacity of macrophages to kill phagocytosed bacteria was not affected by previous virus infection Similar results were obtained when phagocytosis experiments were conducted with macrophages in suspension or when cells were attached to solid surfaces The virus infection had no effect on the opsonic activity of serum The reduction of phagocytic capacity was most pronounced 24 and 48 hours after virus infection thereafter it gradually increased to normal level These data did not correlate with the mortality rate the reduction of elimination of bacteria and frequency of gross pathological changes of the lung The extent of these changes was highest when the infectious agents were given 4 days apart It is questionable therefore whether the antiphagocytic action of the virus upon the alveolar macrophages is the critical factor in the pathogenesis of dual infection From the present data it is not possible to determine the relative importance of inhibition of phagocytosis in the total reduction of host defence after virus infection

The observation of a synergistic effect of combined respiratory tract infection in the mouse with parainfluenza 1 virus (Sendai strain) and *Haemophilus influenzae* b was the subject of a previous report (3) The infection was characterized by a significantly enhanced mortality due to pneumonia as compared with that after infection with either agent alone Elimination of bacteria was slower in animals previously infected with virus Presence of high bacterial concentration in the lung corresponded regularly with clinical illness and with gross pathological changes of the lung No similar association was demonstrated for the infecting virus On the basis of these findings we assumed that a critical factor in the development of dual infection is the local defense mechanism by which the intruding foreign particles are eliminated from the lung

The local host defense mechanism in the respiratory tract is a complex of many factors Foreign particles may be removed through a haematogeneous route by the lymph flow or through the bronchial tree Viable bacteria may be killed by phagocytic cells by the bactericidal action of mucus or by the antibacterial activity of humoral systems Probably the two most important single factors of these are the mucociliary flow trough the trach   ichi and bronchioli

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To the classic concept of a direct spread of staphylococci there must then be added suggestion of a system of very contagious vectors whose precise role remains to be determined

The discussion may be rounded off by two quotations from *Hayes* (1964) (1) One may speculate to what extent we unjustly incriminate bacteria in general for the sins of their viruses and (2) It is strange, in the light of recent discoveries, more attention has not been paid to the possible role of bacteriophages in the conferment of bacterial virulence for man and animals

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scribed earlier (2) Washed cell suspensions containing ca 2–5 × 10 macrophages were pipetted into Leighton tubes in which a flying coverslip covered the entire bottom surface Normal mouse serum was added to make a final 20 per cent concentration The mixture were incubated at 37 C for 18 hours After such treatment the macrophages attached themselves to the surface of glass while other cell types were dead or washed off Bacterial suspension in Eagle Hanks MEM at pH 7 2 was added to make a final ratio ca 100 macrophages per bacterium Normal mouse serum was added in a final concentration of 20 per cent The total volume was 1 ml The tubes were incubated stationary for 1 hour then the surviving bacteria were counted Supernatant fluid was removed and plated in appropriate dilutions The coverslips with the macrophage monolayers were also removed and washed with physiological saline three times in order to remove bacteria not closely associated with the cells A few drops of 5 per cent saponin were pipetted onto the coverslip After a few minutes the cowerships were inverted and rubbed onto the surface of agar media to dislodge any macrophages and bacteria which were still attached to the surface Colony counting was done after 18 hours incubation at 37 C

The difference in numbers of bacteria recovered from test series and control series was attributed to the influence of phagocytic cells The function of these cells was further divided into phagocytic action and intracellular killing shown by the difference in numbers of bacteria recovered from the extracellular fluid and from the destroyed phagocytic cells

Statistical methods Differences in mortality were analysed with a χ^2 test To evaluate the phagocytosis data Wilcoxon s two sample test was used

RESULTS

Since both bacterial strain and mouse strain were different from those employed in previous studies (3) it was necessary to determine whether the synergistic effect could be reproduced with the present system Groups of mice were inoculated with 10^6 egg infectious dose (EID) virus 1–3 × 10^7 colony forming unit (CFU) bacteria or with the combination of both agents and mortality was recorded In the present study mortality after 10 days observation was higher in the *H influenza* infected group (37 per cent) and in the group infected with both agents (83 per cent) than in the previous study (6 and 54 per cent) Mortality after virus infection only was 10 per cent The synergistic effect was also clearly demonstrated with the present system

Phagocytosis by Macrophages from Infected Mice

Macrophages were obtained from mice 4 days after they had received virus inoculation We have demonstrated earlier that the effect of virus infection on bacterial clearance and on gross lungpathology was maximal with 4 days difference between inoculations Wash-outs from 5–7 mice were pooled to make a total 4–8 × 10 macrophages per tube An equal number of cells obtained from normal mice served as controls In the first series phagocytosis was tested with cells in suspension The mean of the results of 6 experiments are shown in Figs 1 and 2 Fig 1 presents the percentage of bacteria recovered from the extracellular fluid The proportion of bacteria eliminated from the experiments are shown in Figs 1 and 2 Fig 1 presents the percentage of bacteria re from virus infected animals were less efficient than cells from normal animals The difference is significant at 30 minutes ($p < 0\ 05$) but not at 60 minutes ($0\ 05 < p < 0\ 1$) Fig 2 gives the percentage of bacteria recovered from the total mixture Elimination of bacteria from the total sample was attributed to the bactericidal action of macrophages Again cells from virus infected mice were less efficient than cells from normal animals The differences are significant (at 30 minutes $p < 0\ 01$ at 60 minutes $p < 0\ 05$) The difference between extracellular and total count is an expression of the bacteria associated with the cells probably phagocytosed but not killed This proportion of the viable bacteria recovered from virus infected cells was slightly larger than that from normal cells however, the difference was not significant $p > 0\ 1$)

In the next series phagocytosis was conducted with macrophages attached to glass surfaces Samples for colony counts were

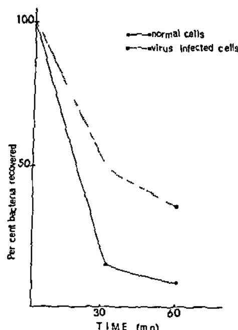


Fig 1 Comparison of the phagocytosis of *Haemophilus influen ae* by suspensions of alveolar macrophages from normal mice and from mice infected with parainfluenza virus The curves are the means of 6 experiments and show the numbers of bacteria remaining in the extracellular fluid

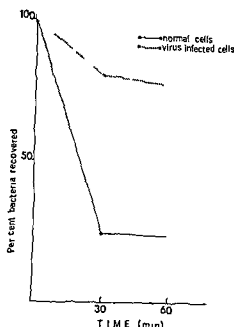


Fig 2 The bactericidal effect of alveolar macrophages from normal and from parainfluenza virus infected mice against *Haemophilus influen ae* The curves are the means of 6 experiments and show the total numbers of bacteria recovered from cells and extracellular fluid

TABLE 1 *Phagocytosis and Intracellular Killing of* Haemophilus influenzae *by monolayers of Alveolar Macrophages from Normal and from Parainfluen a Virus Infected Mice*

Phagocytic cells	Per cent bacteria recovered		
	Extracellular fluid	Macrophages	Total
Normal macrophages	77 8	4 6	8? 6
Virus infected macrophages	92 5	6 6	99 1

taken at the initiation and at the end of the experiments after 1 hour s incubation The mean of the results of 6 experiments are shown in Table 1 Normal macrophages were significantly more efficient than virus infected ones in reducing the number of bacteria in the extracellular fluid and in the total samples ($p < 0.05$) The proportion of bacteria recovered from the macrophages was not significantly different

These experimen s suggest that the phagocytic capacity of i … alveolar macrophages is reduced aft… …nfection with parain'luen… t … with cells from normal mice The ability to kill bacteria already engulfed does not seem to be influenced by the virus infection

Phagocytosis by Cells Infected with Virus in vitro

A large pool of normal macrophages was divided into equal parts and cultured *in* Leighton tubes After the attachment period some tubes were infected with virus other tubes were left uninfected for control By this technique the cell populations in the infected tubes and in the control tubes were

completely homogeneous In pilot experiments phagocytosis was tested 4 days after virus inoculation The infected cell population revealed a reduced phagocytic activity The inhibitory effect was dependent on the concentration of the infecting virus (Fig 3) The minimum concentration which gave detectable effect was 10 EID, increasing to about 100 per cent inhibition when the infecting dose was 10 EID virus In the following phagocytosis experiments the concentration of infecting virus was 10 EID per tube

Effect of Time Interval after Virus Inoculation on Phagocytosis

The development of *in vitro* infection is certainly different from that of *in vivo* infection The four day interval employed in the pilot experiments is probably not comparable to that after *in vivo* infection because the cells are or could be at different phases of the infection In a series of experiments we have investigated the effect of the length of time after *in vivo* and *in vitro* infection on the inhibition of phagocytosis It was also of considerable interest to determine the variation of virus effect on phagocytosis according to the time after infection and its correlation with gross pathology of the lung and bacterial clearance rates when different time intervals between virus and bacterial infections were used

The phagocytic capacity of macrophages at different times after *in vitro* infection was tested and related to the phagocytosis by normal cells originating from the same cell pool The results of a typical experiment are shown in Fig 4 The extent of inhibition at different times varied only slightly but the tendency was consistent The development was characterized by a maximal inhibition 24 to 48 hours after virus inoculation (maximally 26 per cent) thereafter the capacity gradually returned to normal

In the next series macrophages were obtained from infected mice Groups of mice were infected with 10^6 EID virus on consecutive days From 2 hours through 5 days after infection all groups were sacrificed macrophages were obtained and their phago

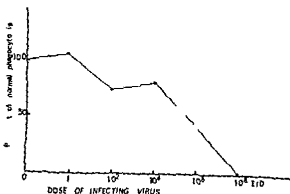


Fig 3 Effect of the dose on the inhibitory action of parainfluenza virus infection on phagocytosis by alveolar macrophages

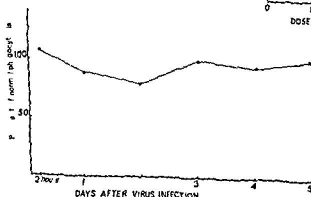


Fig 4 Phagocytosis of *Haemophilus influenzae* by alveolar macrophages at various times after *in vitro* infection with parainfluenza virus

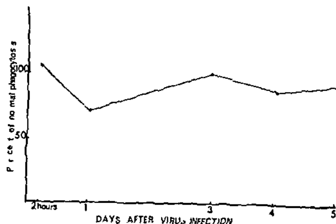


Fig 5 Phagocytosis of *Haemophilus influenzae* by alveolar macrophages at various times after *in vivo* infection with parainfluenza virus

cytic capacity was tested and compared with that of normal control cells The results of a typical experiment are shown in Fig 5 The extent of inhibition at different times after *in vivo* infection was closely correlated with that after *in vitro* infection at corresponding times Maximal reduction was recorded 24 hours after virus inoculation Unfortunately the 2 days samples were not representative in either series completed

The Effect of Time Interval on Mortality

To evaluate further the effect of the length of time between viral and bacterial inoculation mortality rates were determined Groups of mice were infected with virus and at various times from 2 hours through 5 days later with bacteria Mice infected with one agent only served as controls The groups were observed for 10 days and mortality was recorded The results are given in Fig 6 Each group consisted of 36 to 40 mice Compared with the control groups mortality was not significantly enhanced when the combination of infections were given 2 hours apart It was however significantly higher ($p < 0\ 01$) if the bacterial infection followed the virus 1 through 5 days later Among these groups again mortality was significantly higher ($p < 0\ 01$) in the group infected at a 4 days interval when compared with the rest The difference could also be expressed in terms of the length of survival Main survival in days at 2 hours interval 7 51 days at one day 5 04 at two days 4 97 at three days 5 02 at four days 2 02 and at five days 4 05 days In the present system of dual infection the data concerning both mortality and length of survival strongly indicate that the synergistic effect is most pronounced when the agents are inoculated 4 days apart

Effect of Serum Factors on Phagocytosis

In all the experiments described 20 per cent normal mouse serum was included in the mixture Exclusion of serum reduced the phagocytic activity to practically nil Serum from mice previously immunized with and containing agglutinating antibody against *H influenzae* increased the phagocytic activity No difference was observed when the opsonizing effect of normal serum was compared with that of serum from mice immunized with parainfluenza virus

DISCUSSION

The present data indicate that infection with parainfluenza 1 virus inhibits the phagocytic activity of mice alveolar macrophages

Inhibitory effect on the antibacterial activity of phagocytic cells by virus infection has been described earlier Influenza and mumps virus infection reduced the phagocytic action of guinea pig leucocytes (13) *Ginsberg et al* (4 5, 6) also reported inhibition of phagocytosis by guinea pig leucocytes after infection with influenza virus The mechanism of inhibition was postulated

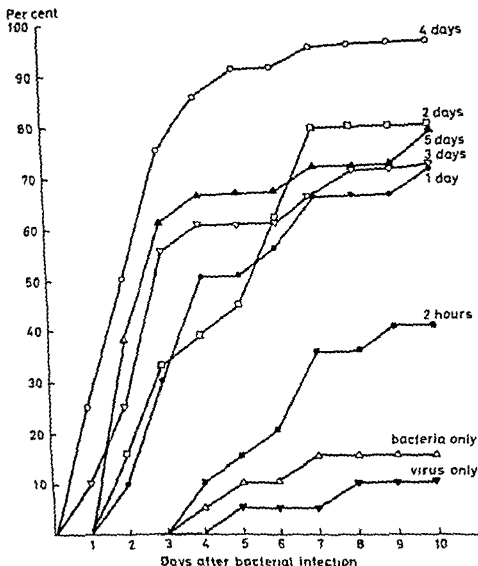


Fig 6 The influence of the interval between inoculation with parainfluenza virus and *Haemophilus influenzae* on the mortality of mice The curves show the cumulative death rates

to be due to a block in the energy yielding mechanisms of virus infected cells Inhibition of phagocytic activity of horse and guinea pig leucocytes by coxackie viruses was observed by *Kantoch & Dubowska Inglot* (10) The effect was due to the action of un inactivated virus associated with the cells and it was dependent on the concentration of the virus and on the length of its action

Some authors also demonstrated an inhibitory effect on phagocytosis during combined infection of the respiratory tract *Wilson et al* (19) investigated the role of humoral and cellular immunity in dual infection of monkeys by influenza virus and *Streptococcus haemolyticus* A depression of phagocytic properties of the circulating granulocytes was demonstrated usually within 24 to 48 hours after virus infection A delay in migration of polymorphonuclear leuco

cytes and slow phagocytic action of pulmonary macrophages during combined infection of mice with influenza virus and pneumococci were observed by *Harford & Hara* (9) Similarly, a reduction of phagocytosis by guinea pig macrophages and granulocytes after influenza virus infection was reported by *Sawyer & Wood* (17) However the significance of their finding for the pathogenesis of the dual infection was doubted *Klein et al* (12) demonstrated that the bactericidal ability of mouse lung was reduced after reovirus infection This change was attributed to an effect on the alveolar macrophage system

The inhibitory effect in the present series could be demonstrated with macrophages when in suspension or when they were attached to solid surfaces We attempted to avoid alternation of the cells employed in the phagocytosis experiments in suspension during preparation They were treated carefully with a minimum of handling and each experiment was completed within 2 hours after removal of the cells from the organism A significant proportion of polymorphonuclear granulocytes was regularly seen in the cell population from virus infected animals These cells were included in the phagocytosis experiments in addition to the macrophages, but only the latter were quantitated We have no data on the extent of participation of leucocytes in the phagocytosis However many of them were non viable by the trypan blue staining method and could not have phagocytic activity If the granulocytes were of considerable significance one would expect an increased difference between normal and virus infected cell populations when the activity of granulocytes are eliminated, e g when macrophages are tested in pure culture The reduction of the phagocytic capacity however was more marked when the cells were in suspension which contained polymorphonuclear leucocytes in addition to macrophages than when cells on glass surfaces were pure cultures of macrophages *Pavlovsky* (15) repo ed that the participation of ˡ ucocyt he phagocytosis of cytoplasmatic inclusions during influenza infection was negligible in comparison with the much higher one of macrophages

The surface technique allowed the performance of phagocytosis experiments with practically pure cultures of macrophages Other cell types were dead or did not attach themselves to the surfaces The process however involved culturing *in vitro* for several hours a process which might alter some of the preperties of the cells Extended culturing reduced slightly the phagocytic activity Controls of identical *in vitro* age were included in all experiments It is doubtful whether the surface phagocytosis phenomenon, described by *Wood* (20), has any significance on the results of the present experiments

The surface technique also made it possible to investigate the effect of *in vitro* infection of cells Although removal of cells from their natural environment might alter their properties it makes it possible to separate the direct action of the virus on the cells from possible indirect mechanisms *In vitro* infection also inhibited the phagocytic activity of macrophages to an extent similar to that in *in vivo* infection Serum opsonic activity was not affected by previous experience with virus The data suggest that the mechanism by which the virus influences the phagocytosis is one acting directly on the phagocytic cells The cellular site of virus action was also pointed out by *Fisher & Ginsberg* (4 5) and by *Kantoch & Dubowska Inglot* (10) The mechanism of inhibition on the subcellular level in the present system remains to be investigated

The length of time between primary and secondary inoculations seems to have critical influence on the development of dual infection Mortality did not increase when the agents were given 2 hours apart as compared with the death rate after infection with either agent alone The mere presence of both agents in the organism does not enhance mortality This indicates that the synergistic effect is a result of a time dependent process Several data indicate that the in

teraction is most efficient when the time interval between primary and secondary inoculation is 4 days It is reasonable to assume that total host defence is maximally reduced about 4 days after virus infection The total host defence is a result of the combination of a number of defence factors The capacity of the defence factor of factors critical in the development of the disease in dual infection is probably maximally reduced at this time The present data indicate that inhibition of phagocytosis is at its largest extent at an earlier phase 24 to 48 hours after virus infection It seems to be questionable therefore whether the reduction of phagocytic activity is the critical factor at least that it is the only one affected A similar conclusion was presented by *Sawyer* & *Wood* (17) using influenza virus as a model The lack of correlation in time makes it necessary to postulate that other local defense factors must be in operation and probably they are critical for the development of combined infection It cannot be ruled out that phagocytosis has an important role From the design of present experiments however, it is not possible to determine how much the inhibition of phagocytic capacity contributes to the total reduction of the capacity of host defence

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cytes and slow phagocytic action of pulmonary macrophages during combined infection of mice with influenza virus and pneumococci were observed by *Harford & Hara* (9) Similarly, a reduction of phagocytosis by guinea pig macrophages and granulocytes after influenza virus infection was reported by *Sawyer & Wood* (17) However, the significance of their finding for the pathogenesis of the dual infection was doubted *Klein et al* (12) demonstrated that the bactericidal ability of mouse lung was reduced after reovirus infection This change was attributed to an effect on the alveolar macrophage system

The inhibitory effect in the present series could be demonstrated with macrophages when in suspension or when they were attached to solid surfaces We attempted to avoid alternation of the cells employed in the phagocytosis experiments in suspension during preparation They were treated carefully with a minimum of handling and each experiment was completed within 2 hours after removal of the cells from the organism A significant proportion of polymorphonuclear granulocytes was regularly seen in the cell population from virus infected animals These cells were included in the phagocytosis experiments in addition to the macrophages, but only the latter were quantitated We have no data on the extent of participation of leucocytes in the phagocytosis However many of them were non viable by the trypan blue staining method and could not have phagocytic activity If the granulocytes were of considerable significance one would expect an increased difference between normal and virus infected cell populations when the activity of granulocytes are eliminated e g when macrophages are tested in pure culture The reduction of the phagocytic capacity however was more marked when the cells were in suspension which contained polymorphonuclear leucocytes in addition to macrophages than when cells on glass surfaces were pure cultures of macrophages *Pavlovsky* (15) repo ed that the partic ipation of l ucocyt he phagocytosis of cytoplasmatic inclusions during influenza infection was negligible in comparison with the much higher one of macrophages

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2 per cent Amino-acids were supplemented in a concentration of 20 μg/ml and the thiamine concentration was 4 μg/ml

EMB plates with a sugar content of 1 per cent were employed except in the case of maltose where the content was 1 5 per cent Ox heart broth infusion was used as complete fluid medium and 1 6 per cent agar was added for use as solid medium. In both cases 1 per cent peptone 0 3 per cent NaCl and 0 2 per cent Na HPO, 12H O were added

In chromosomal transfer experiments made with *Shigella* strains minimal medium was supplemented with 0 025 per cent yeast extract This concentration did not affect the outcome of the experiments

Nomenclature The nomenclature suggested by Demerec et al (9) was followed

RESULTS

Failure to Isolate Stable Hfr Derivatives from an F Population

According to previous observations (15) the most effective chromosomal donor strain among F⁺ *Sh flexneri* strains examined was a derivative of type 4b (D4101) With this strain chromosome transfer was demonstrated for several markers (Thi Thr Trp Ilv Met) with a frequency of about 10⁷ The most useful F tester strains were unexpectedly of heterogenous origin i.e. for the Thi Thr Trp markers *Sh flexneri* 3 (UP3042) and Ilv transfer *E coli* 0100 (D512)

When *Taylor & Adelberg's* technique for Hfr selection was used cultures with an increased capacity of transfer of the Ilv markers were detected Eighty per cent of the F⁺ colonies examined gave Ilv⁺ recombinants on two to three parallel replica plates Some of the F derivatives isolated showed some increase in donor capacity but this phenomenon was not stable since it disappeared on subcultivation One such clone—D4201—showed a remarkably high resistance to acriflavine curing (a loss of the F factor in about 4 per cent in contrast to the curing effect of 70-90 per cent in the control F⁺ strain) However D4201 was able to transfer the F factor with the same frequency as the F⁺ strain (D4101)

Similar results were obtained when the sib selection technique (23) was used Chromosomal transfer was demonstrated in a high percentage of cloned UV irradiated samples of the F⁺ strain D4101, and the frequency patterns of the markers supported the concept of the presence of Hfr derivatives with different integration sites and directions However after purification these phenomena could no longer be observed The UV treatment probably had some effect on the F integration process that might explain the actual chromosone transfer All attempts to isolate *fer* mutants (4) from among recipient populations were unsuccessful The irregularities mentioned might therefore, be caused by defects in the recombination process either in the donor or recipient, or in both

In order to elucidate the role of the recipient cells a study was made of the recombination kinetics in heterologous crosses between K12 Hfr and *Sh flexneri* recipient strains

Interrupted Mating Experiments with Shigella flexneri *3 and 4b Strains as Recipients*

The interrupted mating experiments showed a characteristic pattern in the case of both *Sh flexneri* strains An example is shown in Fig 1 The recombination kinetics of the Thr⁺ Leu (K12 control) Thr⁺ (UP3042) or Leu

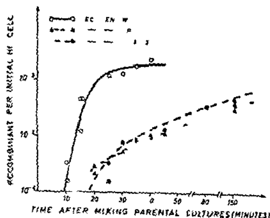


Fig 1 Kinetics of recombinant formation in interrupted mating experiments between Hfr H and the recipient strains of W1177 UP4042 (*Sh flexneri* 4b) and U 30 (*Sh flexneri* 3)

TABLE 1 *Strains Used*

	Designation	Mating polarity	Markers (used)
E coli K12 derivatives	C 8 (HfrH)	Hfr	Thi Str[s]
	C 20 (HfrC)	Hfr	Met Str[s]
	W3287	F_{13} lac	Met
	W1607	F⁻	Met
	W1177	F	Thi Thr Leu Lac Str
	D1177/5	F	Thi Lac Str hsp_{4b}
	D1177/52	F	Thi Lac Str hsp_{4b} *
E coli 0100 K+ H2	D512	F	Hi Ilv⁻
Sh flexneri	WRAIR 2a 691a	Hfr	Lac
Sh flexneri	Type strains 1b 2a 2b 3 4aA 4aB 4b 5 var X and var Y originate from the collection of Institute of Microbiology Pecs and are identical with those used in our previous study**		
Sh flexneri 4b	UP4009	F	Nic Leu Lac Str[s]
	D4101	F	F derivative of UP4009
	D4201	F	Labile Hfr derivative of UP4009
	D4451	F_{13} lac	UP4009 F_{13} lac
	UP4042	F	Nic Leu His Lac Str
	D4042/18	F	Nic His Lac Str[r]hspj
Sh flexneri 3	UP3042	F	Thi Thr His Trp Met Lac Str

* Derived from D1177/5 by acridine treatment

** *Kétyi* (15)

The letters in strain designations mean C (Hirszfeld Institute Wroclaw Poland) W (Lederberg s Wisconsin strains) D (WHO Int Escherichia Centre Statens Seruminstitut, Copenhagen) WRAIR (Walter Reed Army Inst Res Wash) and UP (Inst Microbiol Univ Med Sc Pecs Hungary)

fied recombinants The results were expressed as percentage values

Plasmid transfer The transfer and selection techniques used for F and other transmissible extrachromosomal factors have been described previously (15 16)

Transfer of the hsp genes The K12 *hsp* gene (gene of host restriction and modification of DNA (28)) was transferred by the F_{13} factor carried by the *E coli* K12 strain W3287 The corresponding *hsp* gene of *Sh flexneri* 4b was transferred by means of the F strain D4101 Selections were made for Thr and Leu (K 12 recipient) or Leu (*Sh flexn ri* 4b recipient) recombinants The presence of heterologous *hsp* genes was tested by means of the phage pattern (see below)

Acridine treatment The method des ribed by *Hirota* (14) was empl ed

Phage examination F m n study of the modific on and restri on ns was ma e with phage T5 Designation of modified phage stocks was performed according to *Arber* & *Dussoix* (3) i e T5 3 means the phage stock of T5 prepared with a *Sh flexneri* 3 as host Phage dilutions were made in *Loeb* solution (19) The efficiency of plating (e o p) was determined by the overlayer method (1) The overlayer contained about 10^7 cells of the test strain in 2 5 ml of soft agar Phage stocks were prepared according to *Hershey et al* (13) The results of e o p determinations were expressed in relative numbers taking the arbitrary value 1 for the e o p of a phage assayed on the bacteria that had served as host when the phage was propagated All other values are relative to this figure The quick test according to *Colso et al* (7) was used as preliminary test for the presence of a foreign *hsp* gene in hybrid clones

Media The minimal medium was prepared according to *Falkow et al* (10) but with double concentration of glucose and an agar content of

TABLE 3 *Efficiency of Plating (e o p) of Phage T5 on Different Host Strains of E coli and Sh flexneri*

Host	Relative efficiency of plating				
	Phages				
	T5 K	T5 51?	T5 4b	T5 3	T5 2a
K	1	1	$7\,4\times10^{-}$	$5\,8\times10^{5}$	$5\,0\times10^{3}$
51?	$2\,9\times10$	1	1	$1\,9\times10^{1}$	
4b	$1\,6\times10^{4}$	$1\,1\times10^{-3}$	1	$1\,0\times10^{4}$	$9\,0\times10^{3}$
3	$4\,4\times10^{4}$	1	1	1	$5\,0\times10^{5}$
2a	1		$6\,7\times10^{-}$	$2\,5\times10^{4}$	1

– not tested

Designations of host strains K = *E coli* K12 strain W1177 51? = *E coli* 0100 strain D 51? 4b = *Sh flexneri* 4b strain LP4042 3 = *Sh flexneri* 3 strain UP301? ?a = *Sh flexneri* 2a Hfr 69 WRAIR

The relative values of e o p were calculated on the basis of five independent experiments the figures are arbitrary values relative to the figure 1 given by a phage assayed on the organism that had already served for its growth

latively high fertility of this strain when crossed with *Shigella flexneri* 4b F (15) The relation between K12 and WRAIR Hfr *Sh flexneri* 2a is remarkable since only slight e o p differences were found This finding might be explained by the terminal selection technique used for selection and might throw light on the K12 like behaviour of WRAIR Hfr *Sh flexneri* 2a in our conjugation experiments

It can be mentioned that when T5 was grown either on K12 or coli B the reduction in e o p on the heterologous strain was of the magnitude 10^{4} This is in agreement with the estimation of *Boyer* (5) using lambda

The data concerning restriction and modification between the two *Shigella* strains tested (types 3 and 4b) might have some significance in relation to intra group crosses Thus in the following experimental series e o p determinations were carried out on some *Sh flexneri* type strains with phages T5 K T5 3 and T5 4b The results are recorded in Table 4 Apparantly the degree of restriction is not uniform in different *Shigella* strains For phage T5 K most of the strains tested showed a marked restriction of about 10^{4} On the other hand most of the strains will accept phage T5 4b Phage T5 3 showed strong e o p reductions in five strains and no reduction in the other five It can be concluded from the limited number of experiments that the character of modification and restriction or the specificity of this character varies in different *Shigella flexneri* strains at least when tested with phage T5 DNA The two strains of serotype 2a tested (the WRAIR strain' in Table 3 and the present strain in Table 4) show different restriction and modification thus indicating

TABLE 4 *Efficiency of Plating (e o p) of T5 Variants on Different Sh flexneri Serotypes*

Sh flexneri type trains	Relative efficiency of plating		
	Phages		
	T5 K	T5 4b	T5 3
1b	$5\,0\times10^{4}$	1	1
?a	$3\,?\times10^{4}$	$4\,5\times10^{1}$	1
2b	$1\,4\times10^{4}$	$4\,1\times10^{1}$	$1\,4\times10^{3}$
3	$4\,4\times10^{4}$	1	1
4a ?	$1\,8\times10^{?}$	1	1
4aB	$1\,?\times10$	1	$2\,5\times10^{3}$
4b	$1\,6\times10^{4}$	1	$1\,0\times10^{4}$
5	$1\,6\times10^{5}$	$6\,3\times10$	$1\,1\times10$
Va ?	7×10^{3}	1	1
Var Y	$1\,5\times10^{-}$	1	$2\,8\times10^{3}$
E coli K 12*	1	$7\,4\times10$	$5\,8\times10^{5}$

See legend to Table

* (W1177) [illegible]

TABLE 2 *Linkage Analysis of Recombinants from Crosses between Hfr K12 and F⁻ Strains of K12 Sh flexneri 4b and Sh flexneri 3*

Donor	Recipients	Selected markers	Inheritance of unselected markers (per cent)*		
			Lac	Thr	Leu
AB259	W1177	Thr Leu Str	22.6		
	UP4042	Leu Str^r	25.0		
	UP3042	Thr Str^r	40.1		
	UP4042	His Str	2.2		3.6
	UP3042	Trp Str^r	29.7	4.0	
HfrC	W1177	Thr Leu Str^r	57.5		
	UP4042	Leu Str^r	67.6		
	UP3042	Thr Str^r	64.3		

* *200–300 recombinant colonies investigated*

(UP4042) markers investigated demonstrate marked differences between K12 and *Shigella* recipients. The kinetics of the control crosses K12×K12 was in accordance with the usual finding in such crosses. The appearance of the Thr^+ and Leu^+ markers occurs after 8–10 minutes, then during an interval of about 10–20 minutes the number of recombinants reaches a plateau giving a recombination frequency higher than 10 per initial donor cell. When the two *Shigella* strains are used the appearance of the first recombinants is delayed, there is no plateau formation and there is only a slowly progressing and low recombination frequency reaching values of about 5×10^{-3} after 2½ hours of mating time.

Unexpectedly, similar graphs were given by the above mentioned *Shigella* recipient strains and the Hfr *Sh flexneri* 2a (WRAIR 691a) strain produced by the Walter Reed team (26) using the terminal selection technique. The low recombination rate and late appearance of recombinants were found in this case also. No differences could be found in the linkage analysis (Table 2) between K12 and *Shigella* recipients in the linkage of the unselected marker Lac to the selected Thr or Leu markers. It should be noted that the entry of more distal markers (Lac Trp His) in *Shigella* recipients agrees with data published on map distance in *E coli* K12 (28). These experimental data might be partly or totally explained by the phenomenon of host controlled modification and restriction analogous to that found in *E coli* K12×coli B crosses described by *Boyer* (5, 6).

Demonstration of Host Specific Modification and Restriction by Means of Phage T5

The phenomenon of modification and restriction can be observed by the use of a convenient phage (2). In most of the cases described the phage lambda was employed. This phage could not be used in the present study since *Shigella* strains have no lambda receptor (12). After some trials phage T5 was found to be suitable for the present purpose. The results of e.o.p. determination on the strains used in the above mentioned mating experiments can be seen in Table 3. Marked differences in e.o.p. were found between K12 and *Shigella* strains when tested with T5 grown on K12. The reciprocal tests showed the same pattern with phage T5.3. The restriction of this phage occurred not only in the K12 strain but also in *Shigella flexneri* 4b. Restriction was less pronounced with phage T5.4b when K12 or various other *Shigella* types were used as hosts. Similarly no restriction of phage T5.4b was detected on *E coli* D512, which may explain the re-

TABLE 3 *Efficiency of Plating (e o p) of Phage T5 on Different Host Strains of* E coli *and* Sh flexneri

Host	Relative efficiency of plating				
	Phages				
	T5 K	T5 512	T5 4b	T5 3	T5 2a
K	1	1	7 4 × 10	5 8 × 10^{5}	5 0 × 10^{1}
512	2 9 × 10^{2}	1	1	1 9 × 10^{1}	
4b	1 6 × 10^{4}	1 1 × 10^{2}	1	1 0 × 10^{4}	9 0 × 10^{3}
3	4 4 × 10^{4}	1	1	1	5 0 × 10
2a	1		6 7 × 10	?5 × 10^{4}	1

= not tested

Designations of host strains K = *E coli* K12 strain W1177 512 = *E coli* 0100 strain D 512 4b = *Sh flexneri* 4b strain UP4042 3 = *Sh flexneri* 3 strain UP3042 2a = *Sh flexneri* 2a Hfr 69 WRAIR

The relative values of e o p were calculated on the basis of five independent experiments the figures are arbitrary values relative to the figure 1 given by a phage assayed on the organism that had already served for its growth

latively high fertility of this strain when crossed with *Shigella flexneri* 4b F (15) The relation between K12 and WRAIR Hfr *Sh flexneri* 2a is remarkable since only slight e o p differences were found This finding might be explained by the terminal selection technique used for selection and might throw light on the K12 like behaviour of WRAIR Hfr *Sh flexneri* 2a in our conjugation experiments

It can be mentioned that when T5 was grown either on K12 or coli B the reduction in e o p on the heterologous strain was of the magnitude 10^{-4} This is in agreement with the estimation of *Boyer* (5) using lambda

The data concerning restriction and modification between the two *Shigella* strains tested (types 3 and 4b) might have some significance in relation to intra group crosses Thus in the following experimental series e o p determinations were carried out on some *Sh flexneri* type strains with phages T5 K T5 3 and T5 4b The results are recorded in Table 4 Apparantly the degree of restriction is not uniform in different *Shigella* strains For phage T5 K most of the strains tested showed a marked restriction of about 10^{-4} On the other hand most of the strains will accept phage T5 4b Phage T5 3 showed strong e o p reductions in five strains and no reduction in the other five It can be concluded from the limited number of experiments that the character of modification and restriction or the specificity of this character varies in different *Shigella flexneri* strains at least when tested with phage T5 DNA The two strains of serotype 2a tested (the WRAIR strain in Table 3 and the present strain in Table 4) show different restriction and modification thus indicating

TABLE 4 *Efficiency of Plating (e o p) of T5 Variants on Different* Sh flexneri *Serotypes*

Sh flexneri type strains	Relative efficiency of plating		
	Phages		
	T5 K	T5 4b	T5 3
1b	5 0 × 10^{4}	1	1
2a	3 5 × 10	4 5 × 10^{1}	1
2b	1 4 × 10^{4}	4 1 × 10^{1}	1 4 × 10^{3}
3	4 4 × 10^{4}	1	1
4aA	1 8 × 10^{3}	1	1
4aB	1 ? × 10	1	2 5 × 10^{3}
4b	1 6 × 10^{4}	1	1 0 × 10^{4}
5	1 6 × 10^{3}	6 3 × 10	1 1 × 10
Var X	7 × 10^{3}	1	1
Var Y	5 × 10	1	2 8 × 10^{3}
E coli K12*	1	7 4 × 10	5 8 × 10^{5}

See legend to Table 3

* (W1177)

TABLE 5 *Efficiency of Plating (e o p) of Phage T5 on K12 and* Sh flexneri *4b Hybrids with the Heterologous* hsp *Gene*

Host	Relative efficiency of plating			
	Phages			
	T5 K	T5 K/52	T5 4b	T5 4b/18
K	1	$9 0\times10$	$8 5\times10^{-}$	1
K/52	$2 0\times10^{4}$	1	1	$4 1\times10^{4}$
4b	$3 2\times10^{4}$	1	1	$1 7\times10^{4}$
4b/18	1	$5 3\times10$	$6 0\times10$	1

K = K12 (W1177)
K/52 = K12 with *hsp* gene from *Sh flexneri* 4b
4b = *Sh flexneri* 4b
4b/18 = *Sh flexneri* with *hsp* gene from K12

that this character is strain specific and not type specific

Transfer of hsp Genes and Their Influence on Mating Features

In the course of *Boyer's* studies (5 6) the *hsp* genes of *E coli* K12 and B were found to be allelic and localized near the *thr* region In *Boyer's* experiments, the transfer of a foreign *hsp* gene caused a given host to be able to accept foreign DNA from strains in which the *hsp* originated In our experiments the K12 *hsp* gene was transferred by the F^{13} (lac) factor, Leu^{+} recombinants being selected from the recipient *Sh flexneri* 4b strain UP4042 On the basis of the e o p pattern of phages T5 K and T5 4b, a recombinant designated D4042/18 was chosen for further analysis The reciprocal experiment in which the *hsp* 4b gene was to be transferred into a K12 recipient was unsuccessful when F^{13} was used as fertility factor (perhaps because it is only weakly expressed in the *Shigella* host) However successful experiments were made with an F^{+} (D4101) strain of *Sh flexneri* 4b The presence of *hsp* 4b gene among Thr^{+} Leu^{+} progeny was tested by modified stocks of phage T5 A hybrid strain labelled D1177/5 (F^{+}), was selected This strain had, in addition acquired the F^{+} state and was therefore cured for the F factor by treatment with acriflavine (D1177/52)

The host controlled restriction and modification characters 1 1 brids and of the parent strains are shown in Table 5 It will be seen that the hybrid strains have restriction and modification patterns in relation to T5 which correspond to their parental *hsp* type In addition to the altered phage pattern it should be mentioned that the D1177/5 (F^{+}) culture showed an F transfer capacity to the *Sh flexneri* 4b F^{-} strain of about 12 per cent which is slightly higher than the 1–5 per cent found using the K12 parental donors

In the interrupted mating experiments the above described strains (D4042/18 and D1177/52) and their parents (UP4042 and W1177) served as recipients while Hfr C was

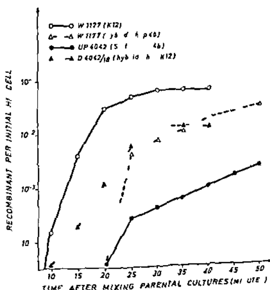


Fig 2 Kinetics of lac+ recombinant formation in interrupted mating experiments between K12 Hfr C and K12 and *Sh flexneri* recipients with initially exchanged *hsp* genes

used as donor. The investigations were limited to Lac+ marker selection. The results are summarized in Fig. 2. Strain D1042/18 which carries *hsp* gene from K12 causes recombinants that appear without abnormal delay. On the other hand the recombination frequency is somewhat lower than that of K12. In the case of the D1177/52 hsp 4b hybrid a late appearance of the first recombinants is found as in coli × *Shigella* crosses. However the recombination frequency is markedly higher than in the case of the *Shigella* recipient. The most probable explanation is that the *hsp* gene and the host specific restriction and modification determined by it play an important role during the conjugational processes. Apparently the influence on recombination frequency is more limited. Thus it is probable that a further factor or factors, e.g. DNA non homology, are responsible for the reduced frequency of recombination.

DISCUSSION

Many authors have reported that the facility with which chromosomal markers were transferred among K12 strains was rarely found when K12 was crossed with other *E. coli* strains or with Enterobacteriaceae strains from other genera. The same infertility was generally found in derivatives of the same non K12 strain when the fertility factor was acquired from K12.

Among the few papers dealing with chromosomal transfer in relation to host controlled modification and restriction, the most important one is that of *Boyer* (5, 6) using *E. coli* K12 and B. The difficulties in plasmid and chromosomal transfer and the failure to detect *E. coli* B Hfr derivatives were found to be the result of host controlled modification and restriction. The problem was solved successfully by the transfer of the K12 *hsp* gene. The transfer of K12 F factor into *Salmonella* can be carried out successfully but recombinants are found only rather infrequently (24). *Baron et al.* (4) isolated so called *fer* mutants from *infer Salmonella* populations. Recently *Okada et al.* (25) published findings which suggested that the *fer* mutants were essentially *hsp* mutants in which the inactivation of foreign DNA does not occur because of the absence of restricting nuclease.

As regards *Shigella* there are only few reports in the literature describing efforts to produce a conjugational system.

Luria & Burrous (20) found reduced frequency of recombination in crosses between K12 and different *Shigella* recipient strains. *Schneider & Falkow* (26) performed crosses between K12 and a *Shigella flexneri* 2a strain and with an Hfr hybrid derivative of the latter prepared by terminal selection. They observed a somewhat reduced (1:10) recombination frequency and a relatively slow conjugation process. On the basis of mating experiments between K12 and *Shigella flexneri* *Czerwinska* (8) concluded that the reduced recombination frequency could not be explained by DNA non homology alone. Isolation of *fer* mutants was not successful.

In the course of our present and earlier (15, 16) studies some irregular features were observed both in the plasmid and chromosomal transfer experiments. These results led to the supposition that the modification and restriction of foreign DNA controlled by the *Sh. flexneri* hosts may have played an important role for these observations. The tests performed with phage T5 seemed to prove this working hypothesis. Further evidence was collected from crosses between strains in which the *hsp* gene had been exchanged. It is thus probable that damage to the plasmid DNA by restriction is responsible for the aberrant recombination kinetics. However the reduced frequency of recombination is probably determined by more factors and the DNA non homology may very well be of significance. Experiments are in progress to ascertain whether it is possible with the help of the K12 *hsp* gene to produce stable Hfr strains from *Shigella flexneri*. When such strains are developed many genetic problems in this group of bacteria will be open for investigation.

TABLE 5 *Efficiency of Plating (e o p) of Phage T5 on K12 and* Sh flexneri *4b Hybrids with the Heterologous* hsp *Gene*

Host	Relative efficiency of plating			
	Phages			
	T5 K	T5 K/52	T5 4b	T5 4b/18
K	1	$9\ 0\times10$	$8\ 5\times10$	1
K/52	$2\ 0\times10^{4}$	1	1	$4\ 1\times10^{4}$
4b	$3\ 2\times10^{4}$	1	1	$1\ 7\times10^{4}$
4b/18	1	$5\ 3\times10$	$6\ 0\times10$	1

K = K12 (W1177)
K/52 = K12 with *hsp* gene from *Sh flexneri* 4b
4b = *Sh flexneri* 4b
4b/18 = *Sh flexneri* with *hsp* gene from K12

that this character is strain specific and not type specific

Transfer of hsp Genes and Their Influence on Mating Features

In the course of *Boyer's* studies (5 6), the *hsp* genes of *E coli* K12 and B were found to be allelic and localized near the *thr* region In *Boyer's* experiments the transfer of a foreign *hsp* gene caused a given host to be able to accept foreign DNA from strains in which the *hsp* originated In our experiments the K12 *hsp* gene was transferred by the F^{13} (lac) factor Leu^{+} recombinants being selected from the recipient *Sh flexneri* 4b strain UP4042 On the basis of the e o p pattern of phages T5 K and T5 4b a recombinant designated D4042/18 was chosen for further analysis The reciprocal experi nt, in which the *hsp* 4b gene was to be ansferred into a K12 recipient was unsuccessful when F^{13} was used as fertility factor (perhaps because it is only weakly expressed in the *Shigella* host) However successful experiments were made with an F^{+} (D4101) strain of *Sh flexneri* 4b The presence of *hsp* 4b gene among Thr^{+} Leu progeny was tested by modified stocks of phage T5 A hybrid strain labelled D1177/5 (F^{+}) was selected This strain had in addition acquired the F^{+} state and was therefore cured for the F factor by treatment with acriflavine (D1177/52)

The host controlled restriction and modification characters I h brids and of the parent strains are shown in Table 5 It will be seen that the hybrid strains have restriction and modification patterns in relation to T5 which correspond to their parental *hsp* type In addition to the altered phage pattern it should be mentioned that the D1177/5 (F) culture showed an F transfer capacity to the *Sh flexneri* 4b F strain of about 12 per cent which is slightly higher than the 1–5 per cent found using the K12 parental donors

In the interrupted mating experiments the above described strains (D4042/18 and D1177/52) and their parents (UP4042 and W1177) served as recipients while Hfr C was

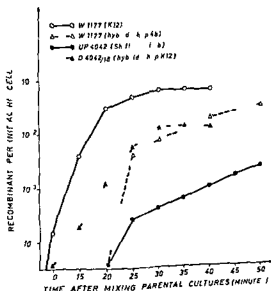


Fig 2 Kinetics of lac+ recombinant formation in interrupted mating experiments between K12 Hfr C and K12 and *Sh flexneri* recipients with initially exchanged *hsp* genes

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CAPSULAR MATERIAL AND MORPHOLOGY OF SOME AMPICILLIN SENSITIVE AND RESISTANT STRAINS OF ESCHERICHIA COLI K12

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In a previous genetic study of a highly ampicillin resistant mutant of *Escherichia coli* it was observed that certain classes of recombinants always showed smooth colony morphology. The present work was undertaken in order to investigate a possible correlation between ampicillin resistance and capsule production giving smooth colony morphology. Two methods for preparing capsular material were compared: ethanol precipitation and negative pressure concentration. Chromatographic examination of these preparations indicated that the negative pressure concentration method gives a more native product with a larger molecular weight than was obtained by the ethanol precipitation procedure. We have compared the amount of capsular material produced by seven different strains, two rough and five smooth. Large variations were observed in the amount of capsule produced but there was no obvious correlation between capsule production and ampicillin resistance. The surface structure of five of the strains was studied by electron microscopy. The smooth strains showed two types of structures closely adhering to the outermost layer of the wall: small vesicles of the order of 40–150 mμ in diameter and minute lamellated units measuring from 80 to 400 Å in length. The rough strains showed very few of these structures. When capsule producing strains were grown at room temperature more capsular material was produced and more of these extra surface structures were observed. It is suggested that the small vesicles and minute lamellated units may represent cell wall material produced in excess amounts.

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Abbreviations: A, absorbancy (subscript for wavelength); EMS, ethyl methanesulfonate; EtOH, ethanol. Abbreviations for genetic markers for which no reference is given in the text may be found in Taylor, A. L. and Dunham Trotter, C.: Revised linkage map of *Escherichia coli*. Bact Revs 31 331–353 (1967).

In recent studies on the genetics of penicillin resistance in *Escherichia coli* K12 it was noticed that *ampA* and wild type strains normally showed rough colony morphology. However, all strains with AmpB phenotype and some other mutants with higher ampicillin resistance always showed smooth colony mor-

TABLE 1 E coli K12 *Strains Used*

Strain	D ampicillin tolerance	Colony morphology	Other relevant markers
D11	1–2	rough	*trp his proB tsx*
D21	15–25	rough	*trp his proB tsx*
D21e7	50	smooth	*trp his proB tsx*
D31	75–100	smooth	*trp his proB tsx*
D51	200	smooth	*trp his proB tsx*
D223	0 5	smooth	*trp proB thy tsx envA*
MC102	2–4	smooth	*leu purE trp capR thi*

All strains were streptomycin resistant The tolerance for ampicillin resistance is the maximum concentration of ampicillin in plates which permits 100 per cent survival of single cells (*Nordstrom et al* 1968)

phology (*Boman et al* 1968) The rough *ampA* containing strain D21 was also the parent strain for the two envelope mutants recently isolated which showed smooth colony morphology and decreased resistance to a number of antibiotics (*Normark et al* 1969) For a study of capsular material we selected the wild type strain, 3 ampicillin resistant and 1 sensitive mutant which all showed smooth colony morphology As controls we included one parental strain with rough colony morphology as well as a mutant known to produce large amounts of capsule For these 7 strains we have compared the amounts of capsular material produced We have also fractionated the material by chromatography on Sepharose 4B and compared ethanol precipitation with negative pressure concentration of the capsule excreted by the bacteria

The morphology of five of the strains was studied by electron microscopy with special emphasis on the outermost surface layers of the bacteria Previously *Work et al* (1966) have correlated lipopolysaccharide production and electron microscopy of the surface layers of some strains of *E coli* *Sapelli & Goebel* (1964) have isolated a capsular polysaccharide from a phage resistant mutant of *E coli* Markovitz group have made a thorough study of the genetics and the regulation of capsule production in *E coli* K12 (for back references see *Markovitz Lieberman & Rosenbaum* 1967)

MATERIALS AND METHODS

Organisms The strains of *Escherichia coli* K12 used are listed in Table 1 Strain MC102 was described by *Markovitz & Baker* (1967) and strains D11 D21 and D31 were described by *Boman et al* 1968 Table 1 also shows the relevant genotypes as well as the phenotypes ampicillin resistance and colony morphology D21e7 is a spontaneous mutant of D21 with properties indicating a mutation of the AmpB type D51 is an EMS mutant of D21e7 which can form single cell colonies on plates with D ampicillin concentrations of 200 µg/ml Strain D22 is a chain forming ampicillin sensitive mutant of D21 fully described elsewhere (*Normark et al* 1969) From this strain was isolated a recombinant (D223) which is thy⁻ and has lost the his gene (*Normark* unpublished)

Medium Minimal medium was made from the basal medium E of *Vogel & Bonner* (1956) supplemented with 0 2 per cent glucose 1 µg/ml of thiamine and the required amino acids (100 µg/ml of the L epimer) The medium for strain D223 also contained thymine (100 µg/ml) and that for MC102 both adenine (100 µg/ml) and a higher concentration of thiamine (10 µg/ml) (*Markovitz & Baker* 1967) MC 102 was grown i 0 4 per cent glucose

Culture and harvesting 1–2 litres of medium was inoculated with 20–25 ml of an overnight culture grown at 37° C on a rotary shaker The cultures were incubated on a rotary shaker either at room temperature (approximately 23 C) or at 37 Growth was followed with a Klett Summerson Photoelectric Colorimeter using filter W66 Growth was interrupted in the late logarithmic phase at 150–180 Klett units corresponding to 1 2–1 4 × 10⁹ cells/ml At harvest a sample was removed for viable count the culture was immediately chilled in an ice water bath the volume

was measured and in order to prevent further bacterial growth sodium azide was added to a concentration of 0 02 per cent The culture was then centrifuged at 20,000 g for 20 minutes in a Lourdes refrigerated centrifuge The supernatant was collected and filtered through a Seitz filter pad in a 4 C cold room To check the effectiveness of the centrifugation and filtration steps test samples without sodium azide were processed in the same manner Viable counts of such filtrates were of the order of 5 × 10⁴ cells per ml Capsular material, as measured by the assay used (see below) was not retained on the filter The filtrate was the starting material for the two following alternative preparative procedures which both were carried out at 4 C

Ethanol precipitation of capsular material To the chilled filtrate was slowly added 3 volumes of 99 per cent ethanol chilled to —20 C during continuous stirring The mixture was allowed to stand overnight in an ice water bath The following day the fine precipitate was collected by centrifugation dissolved in sodium phosphate buffer pH 7 0 ionic strength 0 1 and reprecipitated with 3 volumes of ethanol precooled to —20 C On the third day the precipitate was collected by centrifugation suspended in as small volume of water as possible and dialysed against water After brief dialysis (1–2 hours) the material was in solution Dialysis was continued for 24–36 hours with several changes of water The dialysed capsular material was stored in aliquots frozen at —20 C

Negative pressure concentration of capsular material The filtrate of the culture supernatant was concentrated under negative pressure A piece of dialysis tubing (50–60 cm × 0 64 cm Union Carbide Corporation Chicago Ill) was inserted in a suction flask and by glass tubing through a rubber stopper connected to a reservoir of the filtrate to be concentrated. A tight sleeve of Tygon tubing prevented leakage between the dialysis membrane and the glass tubing Suction was applied with a water pump and the concentration of one litre of filtrate required about 36 hours Capsular material clinging to the walls of the tubing was stripped into the remaining liquid the membrane tied off to form a somewhat tense dialysis bag and the material dialysed for 24 hours against distilled water Like the ethanol precipitated material it was stored in aliquots at —20 C

Chromatography on Sepharose 4B All the chromatographic experiments were carried out using the same column 1 35 cm × 49 cm with a total bed volume of 70 ml Sepharose 4B separates molecules in the range of molecular weights 300 000 to 3×10^6 (*Pharmacia Bulletin* 1967) The column was equilibrated and eluted with sodium phosphate buffer pH 7 0 ionic strength 0 1 containing 0 02 per cent sodium azide recommended as preservative for the Sepharose gel (*Pharmacia Bulletin* 1967) Ascending chromatography was used and a constant flow rate of 4 ml per hour was maintained by a pump Applications were made in volumes of up to 600 µl only for the experiment shown in Fig 5 the volume was 200 µl Eluate was collected in tubes changed every 30 minutes except in the experiment illustrated in Fig 5 when the interval was 20 minutes Chromatography was performed in a cold room at 4 C Determinations of radioactivity were carried out on 0 1 ml samples dissolved in 10 ml of toluene scintillation liquid containing 20 per cent ethanol and counted in a Nuclear Chicago Mark I counter

Assay for capsular material A rapid sensitive method for measuring small amounts of capsular material was provided by the phenol sulphuric acid colorimetric method for determination of reducing sugars and related substances (*Dubois et al* 1956) The colour developed with our capsule preparations had an absorption maximum at 483 mµ the relationship of colour intensity to concentration was linear up to 70 µg/ml. Sodium azide which was used as preservative did not interfere with the reaction The assay was performed with the samples diluted to 1 ml To each sample was added 25 µl of phenol water (80 20 by weigh) followed by 2 5 ml of conc sulphuric acid after each addition the sample was mixed using a Vortex mixer Standard curves were obtained with glucose with soluble starch (no 1252 E Merck Darmstadt) and with capsular preparations from strains D31 and MC102

Electron microscopy Strains D21 D21e7 D31 D51 and MC102 were examined by electron microscopy After growth the bacteria were centrifuged and fixed either according to the method of *Kellenberger et al* (1958) using 1 per cent osmium tetroxide in acetate veronal buffer at pH 6 1 or by the procedure of *Sabatini et al* (1963) using ice cold 4 per cent glutaraldehyde in 0 1 M phosphate buffer at pH 7 3 for two hours In several cases both methods of fixation were employed on the same material After fixation in osmium tetroxide rinsing was performed at room temperature in the acetate veronal buffer for one hour After glutaraldehyde fixation the specimens were rinsed for one hour at 4 C in 0 1 M phosphate buffer containing 0 2 M sucrose They were then post fixed for an additional hour at 4 C in 1 per cent osmium tetroxide dissolved in the sucrose containing buffer (pH 7 3) and thereafter rinsed again this time in distilled water for one hour Dehydration of all specimens was carried out stepwise in ethanol at room temperature and embedding was performed in Epon 812 according to the method of *Luft* (1961) The blocks were cured at 37 C overnight and for an additional period of 24 hours) C

Sections were cut on an LKB Ultrotome using glass knives. Thin sections for electron microscopy were post stained with uranyl acetate and lead citrate and were examined in a Philips EM 300 electron microscope.

RESULTS

The Preparation and Composition of the Capsular Material

According to the literature, ethanol precipitation has been one of the most commonly used methods for the purification of bacterial polysaccharides. In the early phase of this investigation we therefore used ethanol precipitation. However, it was observed that parts of the basal medium often accompanied the polysaccharide in the precipitate and also that the precipitates sometimes were rather difficult to dissolve. Early experiments with the negative pressure concentration method showed that capsular material could be enriched to the degree that the material was a syrup rather than a solution. These concentrated syrups could, however, be mixed with buffers and thereafter handled like homogeneous mixtures. This method was therefore used during the rest of the investigation except when otherwise stated.

Preliminary recording of spectra indicated that the capsular material prepared by negative pressure concentration could not contain any significant amounts of ultraviolet absorbing material such as nucleotides or aromatic amino acids. A Moore and Stein analysis (24 h hydrolysis) of capsular material from strain D31 showed that no amino acid was present in a concentration larger than 0.003 μmoles/mg. No glucosamine or other amino sugars were detected with the amino acid analyzer. The method used for determination of reducing sugar showed that glucose and starch gave the same concentration dependence (Fig 1), while the capsular material from strain D31 gave somewhat higher values.

The amounts of Capsular Material Produced by Different Strains

We observed early that wild type and *ampA* containing mutants showed a rough

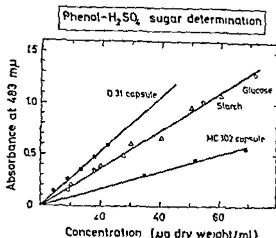


Fig 1 Standard curves showing absorbancy obtained by the phenol-H_2SO_4 sugar determination for varying amounts of material. Glucose (○) and starch (△) were used as known references. The capsular material (●) from strains D31 and MC102 respectively represents the gross material obtained after negative pressure dialysis.

colony morphology while all AmpB containing mutants as well as D31 always gave smooth colonies (*Boman et al* 1968). An attempt to correlate smooth colony morphology and ampicillin resistance was also the basis for the isolation of the chain forming *envA* mutant D22 (*Normark et al* 1969), of which the present strain D223 is a derivative. In the beginning of our investigation we therefore compared the amounts of capsular material produced by different strains. *Markovitz* and his group have isolated and characterized mutants which produce large amounts of capsular polysaccharide. For comparison we have therefore used their strain MC102 which carries the *capR* gene (*Markovitz & Baker* 1967). Table 2 shows that the two rough strains, D11 and D21 produced almost insignificant amounts of capsular material while our other strains produced about 5–50 times more capsular material. Capsule production was usually more pronounced at room temperature than at 37° C. However, at 37° C Markovitz strain MC102, produced about 100 times more capsular material than any of our smooth mutants at 25° C.

TABLE 2 *The Amounts of Capsular Material Produced by Different Strains*

Strain	Growth temp	Culture volume (V)	O D at harvest (K)	Amount of cells (V×K) ×10³	Total dry weight (mg)	Total reducing sugar as glucose (µmoles)	Dry weight (mg) / VK×10⁻⁶	Reducing sugar µmoles/ VK×10⁻⁶
D11	25	888	134	119	0 56	15	4 7	126
D21	25	870	156	136	0 41	10	3 0	74
	37	875	189	165	0 11	13	0 67	79
D21e7	25	875	150	131	10 8	45	8 2	344
D31	25	885	185	164	15 6	71	95	433
	37	860	171	147	3 6	26	24	177
D51	25	800	157	126	3 2	35	25	278
	37	860	208	179	2 2	22	12	123
D223	25	900	169	152	2 5	25	16	164
MC102	37	750	222	167	1234	3017	7339	18066

The medium for MC102 contained 0 4 per cent glucose while all other strains were grown in 0 2 per cent glucose

Gel filtration of Capsular Material

Preliminary fractionation experiments on Sephadex using capsular material from strain D21e7 indicated that the capsular preparation represented a heterogeneous material *and that one component was of very large* molecular weight The penicillinase present in strain D31 has recently been purified (*Lindstrom et al* 1970) and we have also investigated the physiology (*Burman et al* 1968) and the genetics of this strain in some detail (*Boman et al* 1968) We therefore selected this strain for an investigation of the preparative procedure for isolation of capsular material A culture grown at 25° C was harvested at a cell density of 1 4 × 10⁹ cells/ml After centrifugation and filtration the supernatant as divided into two equal parts one was used for preparation of capsule by ethanol precipitation the other was subjected to negative pressure concentration (for details see *Materials and Methods*) Spectra of D31 capsule prepared by these two methods are shown in Fig 2 Since the concentration of reducing sugar in both samples was *almost* the same the spectra indicate that the ethanol precipitated material contained some aromatic material not present in the other preparation Fig 3 shows the chromatogram obtained when *the ethanol precipitated* capsule was investigated on a column of Sepharose 4B Fig 4 shows the chromatogram obtained with the D31 capsular material isolated by negative pressure concentration

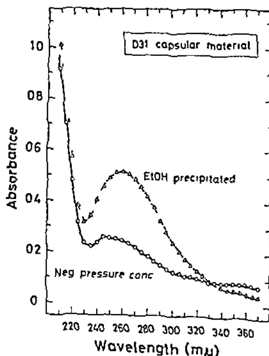


Fig 2 Ultraviolet spectra of capsular material from strain D31 prepared by ethanol precipitation (Δ-----Δ) and by negative pressure concentration (O——O) The concentrations of reducing sugar corresponded to glucose concentrations of 6 09 µmole/ml and 6 55 µmole/ml respectively

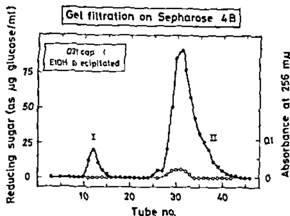


Fig 3 Chromatogram of capsular material from strain D31 obtained by ethanol precipitation The column material was Sepharose 4B The column was pre equilibrated with sodium phosphate buffer pH 7 0 and ionic strength 0 1 containing 0 02 per cent sodium azide Elution was performed with the same agent Quantitative aspects of the chromatogram are given in Table 3 Reducing sugar (●———●) A_5 (O– ––––O)

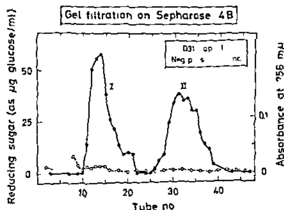


Fig 4 Chromatogram of capsular material from strain D31 prepared by negative pressure concentration The material was obtained from the same culture as used for preparation of the ethanol precipitated capsule investigated in Fig 3 The same column of Sepharose 4B and the same buffer were used as described in the legend of Fig 3 The quantitative aspects of the chromatogram are given in Table 3 Reducing sugar (●———●) A_{256} (O––––––O)

when examined on the same column under conditions similar to those used in Fig 3 Two peaks were obtained in both chromatograms but a comparison shows that peak I which contains l rger molecular weight material was rather small in Fig 3 while it represented about 50 per cent of the eluted material in Fig 4 From the properties of the gel it can be expected that peak I was un retarded material and therefore represents a molecular weight at least of the order of a few millions Judging from the shapes of the curves in Fig 4 the material in both peaks seems to be heterogeneous

The fact that the first peak was almost lost by the ethanol precipitation, but was preserved during the negative pressure concentration indicated that peak I represented a native component of the capsular material However, it could not be ruled out that interaction between the capsular material and the membrane used for concentration produced a polymerization of material from peak II In order to investigate the chromatographic behaviour of untreated capsular material a 1 ml culture of D31 was grown in the presence of 0 1 mC of H 3 glucose The bacteria were removed by centrifugation and the supernatant recovered Equal volumes of this culture medium and capsular material prepared by negative pressure concentration were mixed and applied to the same column of Sepharose 4B The chromatogram in Fig 5

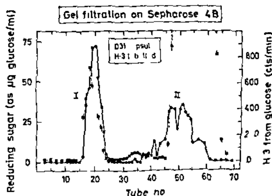


Fig 5 *Chromatogram* of a mixture of the same capsular material from strain D31 (prepared by negative pressure concentration) as used in Fig 4 and an equal volume of medium obtained *from a* culture of *D31* grown *in the* presence of H 3 labelled glucose Note that the fraction volumes were ⅔ of those in the other chromatograms The maximum of the free *glucose* peak was *40 285* cts/min Reducing sugar (●———●) H 3 (■––––––■)

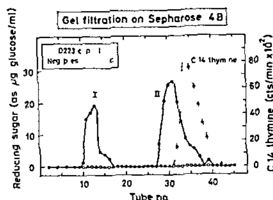


Fig 6 Chromatogram of capsular material from strain D223 prepared by negative pressure con centration The column of Sepharose 4B and the buffer conditions were the same as used in Fig 3 Since the growth medium for strain D2?3 con tains thymine a reference sample of C 14 thymine was added as an internal standard Reducing sugar (●——●) A 56 (O----O) C 14 (▲----▲)

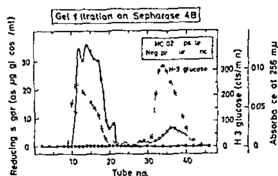


Fig 7 Chromatogram of capsular material from strain MC10? prepared by negative pressure con centration The column of Sepharose 4B and the buffer conditions were the same as used in Fig 3 In this experiment a reference sample of H 3 glu cose was added as internal standard Reducing sugar (●——●) A 56 (O----O) H 3 (■----■)

shows that the first peak which travelled with the void volume contained both radio activity from the glucose and reducing sugar from the capsular material prepared by ne gative pressure concentration This result in dicates that peak I represents a native pro duct and that the alcohol precipitation in some way depolymerized this material The fact that the determinations of reducing sugar and the radioactivity do not coincide in peak I indicates that the material is he terogeneous The second peak contains the large amount of unused glucose from the me dium which overlaps the second peak of the capsular material to such an extent that no conclusions can be drawn concerning this material

The negative pressure concentration meth od was also used to prepare capsule from the *ent* 4 containing strain D223 and from Mar kovitz strain MC102 The chromatograms in Figs 6 and 7 show that both strains pro duced material of the peak I type However, MC102 showed no peak II with reducing sugar while D223 produced the lower mole cular weight material as well To the capsular material from strain D223 (which requires thymine) was added a sample of radioactive thymine to the MC102 capsule a corre sponding amount of radioactive glucose Figs 6 and 7 do not show any radioactivity in peak I which indicates that the thymine and the

TABLE 3 *Recovery from Gel Filtration on Sepharose 4B*

Capsule	Reducing sugar as glucose (µmoles) *Applied*	*Recovered* Peak I	Peak II	Per Cent Recovery Peak I	Peak II	Total
D31 negative pressure concentrated	8 6	2 7	2 5	31	29	60
D31 EtOH precipitated	8 0	0 5	4 6	6	58	64
D223 negative pressure concentrated	3 6	0 7	1 3	19	3b	55
MC10? negative pressure concentrated	4 1	2 6	<0 0?	63	<? 5	63

glucose present in the growth medium were completely separated from the polysaccharide material present in this part of the chromatogram In Fig 6 the main part of peak II travelled ahead of the thymine and the glucose peak in Fig 7 started in the same place in the chromatogram This represents only a partial separation and peak II may therefore not be free from medium components

Since the capsular material is quite viscous and poorly soluble it was considered important to calculate the recovery of the material applied in the chromatograms shown Table 3 summarizes the recoveries in the gel filtration experiments shown in Figs 3, 4, 6, and 7 The total recovery varied from 55–64 per cent which shows that some material had been retained on the column

Quenching of Azide Spectra by Capsular Material

In order to prevent microbial growth it is recommended that Sepharose gels should be kept in solutions containing 0 02 per cent sodium azide (*Pharmacia Bulletin* 1967) Our centrifugation and filtration procedure only reduced viable counts to a level below 10^5 To prevent growth of this residue of bacteria we routinely added azide to all capsular preparations However, azide has a significant absorbancy at wave lengths below 260 mμ and it was accidentally found that capsular material could quench part of this absorbancy The mechanism of this quenching is not known to us We have, however used this finding to test for qualitative differences between the capsular material produced by different strains as well as for discriminating between the material present in peaks I and II from the Sepharose fractionations Quenching was recorded from about 250 mμ down to about 200 mμ which was the limit set by the instrument We arbitrarily selected 280 and 240 mμ as wave lengths and have recorded the differences between a sodium phosphate buffer (pH 7 0 ionic strength 0 1) containing 0 02 per cent sodium azide and fractionated capsular material dissolved in the same solution The results in Table 4 show that all materials tested gave quenching at 240 mμ but with a 40 fold variation In agreement with the results in Figs 2–4 the azide quenching indicates that ethanol precipitation changes the capsular material For material prepared by negative pressure concentration peak II shows significantly higher quenching than found for peak I

The Morphology of the Surface Layers of Capsule and Non Capsule Producing Strains

An attempt was made to obtain electron microscopic pictures of the capsular material

TABLE 4 *The Quenching Effect of Fractionated Capsular Components on Azide Spectra*

Capsular component	Strain	Prep of capsule	Reducing sugar as glucose μg/ml	ΔA_{280}	ΔA_{240}	Normalized to 15 μg glucose/ml ΔA_{280}	ΔA_{240}
	D31	Neg pressure	132	0 069	—0 180	0 008	—0 0?
Peak I	D31	EtOH precip	40	0 051	—0 156	0 0?	—0 06
	D223	Neg pressure	31	0 036	—0 08?	0 0?	—0 04
	MC10?	Neg pressure	166	0 103	—0 200	0 009	—0 0?
	D31	Neg pressure	23	0 0?2	—0 233	0 014	—0 15
Peak II	D31	EtOH precip	4	0 142	—0 010	0 53	—0 04
	D??3	Neg pressure	8	0 034	—0 506	0 06	—0 95

The absorbancy of 0 02 per cent sodium azide in phosphate buffer at pH 7 0 was 0 965 at 240 mμ and 0 012 at 280 mμ ΔA_{280} and ΔA_{240} are the absorbancies of the capsular components in this solution read against the latter as blank The normalized values assume that quenching was proportional to content of reducing sugar

prepared by negative pressure concentration of medium from strains D31 and MC102 as such However, the low density of the material made it difficult to carry it through the preparatory procedures for electron microscopy No characteristic morphological structures were observed in this material If originally present they may well have been lost during fixation and embedding

However, an electron microscopic comparison of the strains investigated yielded information concerning the fine structure of the surface layers of the bacteria Mainly two types of structures were observed small vesicles and minute lamellated units It should be pointed out that these structures were best preserved and showed up to greatest advantage after fixation in glutaraldehyde and postfixation in osmium tetroxide

The vesicles varied somewhat in diameter from 40 to 150 mμ and were observed both attached to the cell surface and lying free in the close vicinity of the bacteria (Figs 9–13) They were delimited by a characteristic triple layered structure consisting of two electron dense lines and an intervening space of lower density The overall thickness of this delimiting structure was of the order of 75–100 Å The vesicles were mostly filled with a fairly electron dense amorphous material (Figs 9 and 11) The lamellated units measured from 80 to 400 Å in length (Figs 11 13) They often showed a triple layered structure consisting of two electron dense layers measuring about 25 Å across separated by a roughly 50 Å thick, electron lucid layer Frequently however several such units were fused into quintuple or multi layered structures (Fig 13) The lamellated units were often attached to the bacterial surface from which they projected at various angles In many locations they appeared to be intimately associated with the well defined triple layered outermost component of the cell wall Figs 8 and 9 are electron micrographs of the rough strain D21 grown at 37° C and 25° C respectively Only occasionally and chiefly at 25° C were a few vesicular structures (Fig 9)—and very rarely some lamellated units—observed close to the surface of the cells of this strain These observations should be compared with the results in Table 2 which show that at neither temperature did this strain produce significant amounts of capsule

Fig 10 shows an electron micrograph of strain D21e7 grown at 37° C and Figs 11 and 12 of D31 grown at 25° C and 37° C, respectively Pictures of these two strains as a rule show both vesicles and lamellated units either directly associated with the outermost layers of the envelope or lying relatively close to the bacteria These structures were most frequent in D31 after growth at room temperature (Fig 11) This is consistent with the results in Table 2 which show that at 25° C strain D31 produced considerably more capsular material than D21e7 (Fig 10) and about 3 times the amount it produced itself at 37° C (Fig 12) Our most resistant mutant strain D31, shows considerable variation in cell size A relatively large number of cells exhibit about two three times the normal length found for strains D21 and D31 Also with this mutant, grown at 37° C the surface layers showed numerous vesicular structures and lamellated units closely adhering to the outermost layers of the wall (Fig 13) The chain forming mutant D223 showed relatively few vesicular structures and may represent a special case It is hoped to treat its morphology elsewhere in connection with a study of the division mechanism

Strain MC102 is clearly filamentous and thus quite different from all our strains Furthermore, despite similar treatment in all respects this strain did not show either the vesicles or the lamellated units found in our capsule producing strains However, from the surface of cells of this strain numerous strands of an amorphous material projected into the surrounding medium for distances up to several microns (Fig 14) Both qualitatively (Fig 7) and quantitatively (Table 2) capsule production of this strain is different from that of our related set of strains



Fig 8 Strain D21 grown at 3 C A normal cell division is seen No specific structures are observed extracellularly Electron micrograph × 31 000
Fig 9 Strain D21 grown at 25 C A few vesicular structures are observed one of which is intimately associated with the outer component of the cell wall Electron micrograph × 113 000
Fig 10 Strain D21 7 grown at 37 C Vesicles and lamellated units are present *but only* in small numbers Electron micr × 55 500



Fig 11 Strain D31 grown at 25 C Vesicles as well as numerous lamellated units are observed around the peripheries of the cells Many of the lamellated units are jutting out from the surface at various angles Electron micrograph × 8? 250







Fig 12 Strain D31 grown at 37 C This electron micrograph shows both lamellated unit and vesicular structures One of the latter (centre of the picture) appears to be closely related to the outer component of the cell wall × 79 350

Fig 13 Strain D51 grown at 37 C Numerous vesicles and lamellated units are seen in c ose relation to the bacterial cell wall Many of the latter are stacked upon each other forming multilayered structures Electron micrograph × 101 800

Fig 14 Strain MC 102 grown at 25 C Vesicles and lamellated units are not seen However at the cell surface numero is projections of some apparently amorphous substance may be observed Electron micrograph × 64 700

DISCUSSION

Definition of Capsule

Davis et al (1967) define bacterial capsule as "a loose gel like structure which varies widely among strains in thickness density and adherence to the cell wall". This statement also indicates the experimental difficulties involved in defining a bacterial capsule. If bacteria in a culture are removed by centrifugation only part of the capsule may remain in the medium. On the other hand the method of heating the culture to 100° C for some minutes (*Markovitz & Baker* 1967) may permit some parts of the envelope to be lost from the bacteria and to contaminate the capsule. A heating step can furthermore be expected to change delicate chemical structures present in the native material.

We have in the present report limited ourselves to the study of capsular material which readily leaves the bacteria of a culture grown with normal agitation. Centrifugation followed by filtration was found to be the best method for removing the bacteria. Even with this limitation the capsule preparation must meet the following requirements: (a) The amount of bacteria must be below a given value and further growth should be stopped. (b) Components added to the medium for supporting the bacterial growth should be separated from the capsule synthesized and excreted by the bacteria.

That requirement (a) was met is indicated by the viable count which after centrifugation and filtration always was less than 10^5 cells/ml. To prevent further growth azide was present both during the rest of the preparation as well as during the chromatography. The fact that our capsule preparation from strain D31 was free of amino acids is additional evidence that the contamination with whole bacteria has been negligible.

Requirement (b) cannot be said to have been rigorously met by the fact that all preparations were dialyzed against water and the knowledge that all components of the minimal medium are known to pass dialysis membranes. However, Figs 6 and 7 show that the large molecular weight components present in peak I were free of the labelled medium compounds added. That the ethanol precipitation affected peak I was shown by comparing Figs 3 and 4. The experiment with the untreated culture fluid containing capsule labelled with H^3 glucose showed that peak I material was present (Fig 5). It can therefore be concluded that peak I in our chromatograms represents a native capsular product of very large molecular weight.

The present data do not permit safe conclusions about the material in peak II but it seems likely that at least part of this material is excreted by bacteria. However, other separation methods will have to be applied for at further characterization of peak II.

Capsular Material from Different Strains

Despite the fact that the recoveries in the gel filtration experiments were not near 100 per cent (Table 3) one can probably conclude that the capsular material of strain D31 is different from that of D223. The capsular material of strain MC102 is clearly different from that of the two other strains. This is indicated by the content of reducing sugar (Fig 1) by the absence of peak II and by the fact that peak I in this case contains significant amounts of UV absorbing or light scattering material which travels before the peak of the reducing sugar (Fig 7). The azide quenching (Table 4) also indicates that the capsular material is quite different in strains D31, D223 and MC102.

In connection with their work on the capsular material produced by a phage resistant mutant and by a colicine K producing strain *Sapelli & Goebel* (1964) reviewed the earlier literature on *E coli* capsular polysaccharides. However, most of these investigators have used alcohol precipitation a step also used by *Leive & Shovlin* (1968) in their investigation of a lipopolysaccharide from the surface layers of *E coli* by *Adams & Young* (1966) who isolated a







Fig 12 Strain D31 grown at 37 C This electron micrograph shows both lamellated units and vesicular structures One of the latter (centre of the picture) appears to be closely related to the outer component of the cell wall × 79 350

Fig 13 Strain D51 grown at 37 C Numerous vesicles and lamellated units are seen in close relation to the bacterial cell wall Many of the latter are stacked upon each other forming multilayered structures Electron micrograph × 101 800

Fig 14 Strain MC102 grown at 25 C Vesicles and lamellated units are not seen However at the cell surface numerous projections of some apparently amorphous substance may be observed Electron micrograph × 64 700

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VIRULENCE AND IMMUNOGENICITY OF MUTANT STRAINS OF *SALMONELLA TYPHIMURIUM*

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The virulence of R mutants derived from the S strain *Salmonella typhimurium* 395 MS has been investigated and compared with their phage pattern chemical composition of the lipopolysaccharide agglutination by O specific serum and absorption of O specific anti bodies No strict relationship between phage pattern or chemical composition and virulence could be established The most virulent mutants (R4a R9) seemed to have O specific antigen at their surface whereas no O specific antigen could be demonstrated in the least virulent one (R10) which also showed strong spontaneous agglutination Two rough resistant mutants R5 and R6 with large quantities of O specific antigenic determinants at their surface showed low and almost no virulence respectively Mucin reduced the lethal dose considerably the factors ranging between 10^{2} and 10^{5} Mice surviving for 5 weeks after infection often demonstrated a chronic disease with enlargement of the spleen and sometimes yellow white patches in the spleen and in the liver Mice surviving doses of R bacteria that might be lethal to some animals in the same group often survived challenge with S bacteria Particularly R9 a galactose-4 epimerase less mutant gave a high degree of protection in surviving mice

In order to confer immunity to *Salmonella typhimurium* in mice active immunization with the O antigen, particularly Salmonella factor 0–5 and passive immunization with purified anti 0–5 antibodies have been ade quate (*Jackson Rowley & Jenkin* 1968) The O antigen determinant is the distal part of the cell wall lipopolysaccharide (LPS) in S bacteria and constitutes at least part of their cell surface The synthesis of the O antigen determinant however takes place deeper in the cell and via a lipid intermediate in the cytoplasmic membrane it is incorporated in the cell wall LPS (*Horecker* 1966) However, certain R mutants which have a deficient core (see Fig 1) and intact synthesis of the O antigen determinant do not put together an O antigenic LPS but produce the O anti gen determinant such that it after extraction appears as a separate hapten (*Beckmann Sub baiah & Stocker* 1964)

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With a series of mutants available which had been characterized with regard to chemi cal composition and immunochemistry of the LPS and to phage pattern (*Holme et al* 1968 *Lindberg & Holme* 1968) this investigation was started to analyse the effect of the quanti ty nature and location of the O antigen de terminant on the virulence and immuno genicity for mice Several investigators have stressed the necessity of living cells for the de velopment of immunity against Salmonella (*Roantree* 1967 *Kurashige et al* 1967) although this opinion has been refuted by others (*Jenkin & Rowley* 1963)

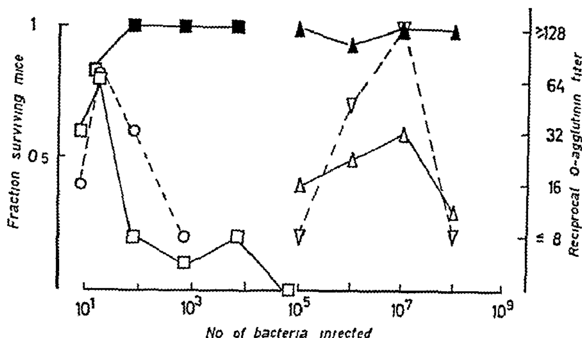


Fig 5 S typhimurium R9 injected intraperitoneally into mice R9 bacteria suspended in mucin □ fraction of surviving mice ○ 0 agglutinin titre ■ fraction of mice surviving challenge with S bacteria R9 bacteria suspended in saline △ fraction of surviving mice ▽ 0 agglutinin titre ▲ fraction of mice surviving challenge with S bacteria

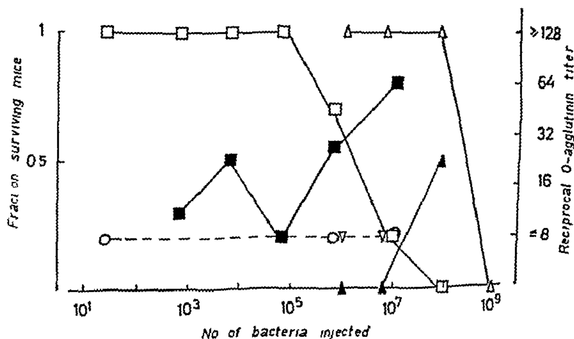


Fig 6 S typhimurium R10 injected intraperitoneally into mice R10 bacteria suspended in mucin □ fraction of surviving mice ○ 0 agglutinin titre ■ fraction of mice surviving challenge with S-bacteria R10 bacteria suspended in saline △ fraction of surviving mice ▽ 0 agglutinin titre ▲ fraction of mice surviving challenge with S bacteria

Antibody Response

The antibody response towards the O antigen determinant of mice injected with the R mutants varied with the mutants (Table 1) About half of them (R5 R6 R7, R8 and R10) gave no significant titres, whereas R4 and R9 gave significant titres in all tested sera

When mice injected with R bacteria were challenged with 10^6 S bacteria some animals survived (Figs 2–6) After the higher doses of the R mutant greater fractions of the mice survived the challenge In these cases however usually so many mice were killed by the R bacteria that the actual number of mice alive after challenge with S bacteria was small One exception is R9 where almost all mice injected with this mutant survived the challenge At autopsy of animals killed at the end of the experiment the spleen was often enlarged sometimes the spleen and the liver showed yellow patches and in rare cases there were adhesions in the peritoneal cavity Autopsy cultures often gave growth of *S typhimurium* either S bacteria the injected R mutant or both of them Titration of sera from mice surviving challenge with S bacteria always gave titres higher than 1 128

Several of the R mutants were positive in slide agglutination with Salmonella factor 0–4 serum (Table 1) When no agglutination occurred in Salmonella factor 0–9 serum non specific agglutination was ruled out All R strains but not the S strain were sensitive to normal human serum Only R9 did not ferment galactose

Antibody Absorption by Heat Killed Bacteria

The absorption of antibody by the different strains was similar with the Salmonella factor 0–4 serum and the antiserum against an ether extract from an S strain *S typhimurium* LT2 (Table 2) The strains 395 MS R5 and R6 had the highest absorbing capacity One absorption was sufficient to remove all or almost all haemagglutinating activity even from the highest concentrations R4a showed a smaller but still evident absorption With R1 there may be some absorption, whereas the reduction in titre with the remaining strains might be due to a dilution effect and non specific adsorption

TABLE 2 *Absorption of Agglutinins Tested with Passive Haemagglutination with S Lipopolysaccharide by Different* S typhimurium *395 M Mutants*

S typhimurium 395 M mutant used for absorption	Antisera against S typhimurium 0 antigen used for absorptions															
	anti S LPS 26 HAU				anti S LPS 256 HAU				anti 04 16 HAU				anti-04 128 HAU			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
S					0	0	0	0	0	0	0	0	0	0	0	0
R0	8	4	2	1					8	4	4	1	>8	>8	>8	>8
R1	2	0	0	0	>8	>8	8	4	8	4	4	2	>8	>8	>8	>8
R2	8	8	4	2					8	4	2	0	>8	>8	>8	>8
R3	8	2	0	0					8	4	1	0	>64	>64	>64	>64
R4a	4	0	0	0					2	0	0	0	>64	64	64	4
R5	0	0	0	0	0	0	0	0					2	0	0	0
R6	[illegible]	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
R7	4	2	1	0					4	1	0	0	>8	>8	>8	>8
R8	4	2	1	0					8	4	4	0	>8	>8	>8	>8
R9	8	2	1	0					16	16	8	>4	>8	>8	>8	>8
R10	4	[illegible]	2	1					8	8	4	4	>8	>8	>8	>8

An antiserum prepared by injection of an ether extract of S typhimurium LT 2 (an S-strain) intravenously into rabbits was used in concentrations of 26 and 256 haemagglutinating units/0 2 ml 'An anti factor 0–4 serum was used at 16 and 128 HAU/0 2 ml Four absorptions were made with each serum

DISCUSSION

During the last decades considerable information has been gathered about the genetics (*Subbaiah* & *Stocker* 1964, *Stocker Williamson* & *Makela* 1966), the biosynthesis (*Horecker* 1966) and the chemical structure and immunochemistry (*Lüderitz Staub* & *Westphal* 1966 *Lüderitz Jann* & *Wheat* 1968) of the cell wall lipopolysaccharides (LPS) of Salmonella, particularly of *S typhimurium* Within this species mutants with well defined carbohydrate deficiences in the structure of their cell wall lipopolysaccharide are known However few investigations have been made concerning the virulence and immunogenicity of such well defined mutant strains

The R mutants suspended in saline displayed a wide variation of virulence when injected into mice (Figs 2–6 Table 1) This variation did not seem to be related to phage pattern or chemotype The effect of the O antigen determinant is more difficult to evaluate *Holme et al* (1969) suggested that at least some of the mutants *S typhimurium* 395 MR0–R10 might have incomplete blocks which would lead to a defective synthesis of the O antigen In our experiments the most virulent mutant, R4a, was positive in slide agglutination with Salmonella factor 0–4 serum and showed a clear cut absorption in the lower concentrations of antibody From these data it seems likely that R4a has such a defective O antigen synthesis and the other mutants with intermediate virulence might be similar (leaky mutants) The next virulent mutant R9 was positive in slide agglutination but did not show a distinct absorption R9 is galactose epimerase less which means that its content of O antigen is markedly influenced by galactose In comparison, R5 and R6 the latter being nearly non virulent were the mutants that showed the greatest absorption of all the mutants R4a and R9 are sensitive to the Felix 0–1 phage which implicates that their cell wall LPS contain the complete core (*Lindberg* 1967) O antigen determinants present in [illegible] cell walls have the prerequisites to ev[illegible] the compl te O antigen LPS In contrast, R5 and R6 are of the rough resistant type The cell walls of similar mutants contain the O antigen determinant largely as O specific hapten free from the LPS core (*Kent* & *Osborn* 1968) Since intact bacteria of their Rb strain (TV 227) did not adsorb the O specific phage P 22 but did so after spheroplasting they concluded that the O specific hapten was localized beneath the cell surface Earlier investigations (*Lindberg* & *Holme* 1968) have shown that, among of the R mutants investigated the cell wall LPS of R5 and R6 contain the largest amounts of O specific sugars and that LPS from R5 cross reacted with O factor 4 In a later communication *Holme* & *Lindberg* (1969) demonstrated the O specific determinant of R5 both as LPS and as hapten In R6 only the hapten was described The hapten was also found in R3 (= Ra), R1, R7 and R8 (= Rb) and R10 (= Rd) No certain absorption of O specific antibody was displayed by these mutants in our experiments however Since in our tests also living and UV killed R6 bacteria absorbed antibody evidence exists that the O antigen determinant occurs at the surface of the very low virulent mutant R6 Further experiments are required to show the nature of the O specific determinants in the cell walls of R5 and R6 The observation that injection in mice of R5 or R6 did not elicit O antibody production whereas R4a and R9 did further indicates that most of the O specific determinant in R5 and R6 is haptenic

Addition of mucin to the bacterial suspension increased the virulence of all mutants substantially The most virulent mutants attained the same killing dose as the S bacteria in saline When mice surviving such treatment were killed and autopsied signs of chronic infection were often observed and the injected R mutant could be isolated from affected organs although the mice seemed to be in good condition These observations indicate that these R mutants are capable of surviving intracellularly which has been shown for other R mutants (*Roantree* 1967) The function of the O antigen could then be to protect the

cteria outside the phagocytes These experi ents suggest that mucin had a similar func on for the R mutants which has been pro osed earlier in other connections The exact unction of mucin is however, not known Olit ki 1948 *Wilson & Miles* 1964)

A preceeding infection with an R mutant ave in some cases protection against chal enge with 10 S bacteria, a dose which is 0⁴–10 times the dose known to kill almost ll control mice A particularly high degree of protection was obtained in mice surviving loses of R mutants which were lethal to some mice in the same group No protection was obtained in mice however if 0 3 mg of the same heat killed mutants was used (*Holme et al* 1968) Since the PD 50 of the smooth form was 6 × 10 mg the immunogen was probably not destroyed by the heat treatment Rather the quantity of O specific antigen was not sufficient to provoke a protective immune response *Kenny & Her berg* (1968) who worked with a mouse *S typhimurium* system with an LD 50 of 5 × 10⁴ bacteria and a challenge dose of 100 LD 50 found a good correlation between the presence of specific bactericidal antibodies in serum and immunity In addition a non specific immu nity of shorter duration was observed *Col lins* (1968) has further stressed the import ance of a non specific kind of immunity to Salmonella which is not dependent on con ventional humoral antibody but connected to an existing infection Our experiments showed that, when antibodies were demon strated protection existed With some mu tants protection was established although no antibodies were found This protection can be due to antibodies not detected by our method or to another mechanism of immunity

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REISOLATION OF *MICROCOCCUS MUCILAGINOSUS* MIGULA 1900

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In cultures from the pharynx one frequently observed Gram positive catalase positive cocci with characteristic transparent to grayish white colonies with a coherent consistency These often adhered firmly to the agar surface Eighteen such strains including one from bronchial secretion and three from blood cultures were subjected to detailed biochemical serological and pathogenicity studies They were also examined for lysostaphin sensitivity and the DNA base composition was determined All the strains appeared as members of the same species On the basis of a careful review of the literature we found that this species had been named *Staphylococcus salivarius* Andrewes & Gordon 1907 and was reisolated by *Gordon* (19) However the per cent (G + C) of these strains their lysostaphin resistance and lack of anaerobic glycolysis are compatible only with the genus *Micrococcus* The strains are not identical to *Gaffkya tetragena* *Diplococcus crassus* *Micrococcus lyicus* or *M freudenreichii* On the other hand they possess all the properties of *M mucilaginosus* Migula 1900 which is therefore the earliest available epithet for this species and must be adopted Micrococci have been reported to be typically novobiocin resistant Neither the strains of *M mucilaginosus* nor those of *M freudenreichii* examined by us adhered to this concept *M mucilaginosus* is important for its frequency of occurrence in cultures from the human pharynx mostly without clear relation to disease The possibility of potential pathogenicity in man cannot be decided

In specimens from the pharynx our attention has been directed towards frequent mucoid semitransparent to opaque colonies of a characteristic coherent consistency often firmly adherent to the surface of the blood agar The identity of these microbes was not readily evident but by modern criteria (4 5 39) they were considered to belong to the family *Micrococcaceae* Pribram 1929 Their characteristics were compatible with a species described by *Migula* in 1900 (27)

The purpose of this paper is to present an extended description of this species report lysostaphin susceptibility and DNA base composition and demonstrate the relationship to previously named species and to genus

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MATERIALS AND METHODS

Strains Most strains were isolated from throat swabs except for three strains isolated from blood cultures and one from bronchial secretion Their designations and origin are listed in Table 1

For comparison it was desirable to know the detailed characteristics of the following type collection strains which were therefore studied to the same extent as the strains collected by us

TABLE 1 *Designation and Origin of Strains*

Designation	Origin	Patient diagnosis
5762/67	Throat	Sinusitis
421/68	Throat	Rhinitis
623/68	Throat	Rhinitis allergica
767/68	Throat	Rhinitis
789/68	Throat	Rhinitis
795/68	Throat	Tonsillitis
1124/68	Throat	Respiratory tract infection
2843/68	Throat	Pharyngitis chronica
2849/68	Throat	Lip ulcer with *Candida* sp
2852/68	Secretion obtained through bronchoscope	Infiltr pulm
S 3878/68	Throat	Tumor colli
S 3938/68	Throat	Otosclerosis
S 4082/68	Throat	Cancer laryngis
S 4239/68	Throat	Hypertrophia conchae nasi
S 5269/68	Throat	Sarcoma orbitae
MA 4399/69	Blood	Abscessus pulm Diabetes mellitus
MA 6903/69	Blood	Insuff valv aortae operat
KA 7178/69	Blood	Seclusio foram sept ventric

1 CCM 547* *Micrococcus conglomeratus* (= ATCC 401 NCIB 2677 NCTC 2677)
2 CCM 810 *M luteus* (= ATCC 398 NCIB 8165 NCTC 8512 proposed neotype)
3 CCM 679 *M roseus* (= ATCC 186 NCTC 7523 proposed neotype)
4 NCTC 7281 *M varians* (currently carrying the name *Staphylococcus lactis* in NCTC but originally called *M varians*)
5 CCM 764 *M freudenreichii* (= ATCC 407)
6 ATCC 407 *M freudenreichii* (NCTC 2679 discarded from the Collection in 1958)
7 CRIPP A 6 *M freudenreichii*
8 CRIPP A 171 *M freudenreichii*

* ATCC = American Type Culture Collection Rockville Md CCM = Czechoslovak Collection of Microorganisms Brno FDA = Food and Drug Administration Washington D C U S A CRIPP = Central Research Institute of Plant Production Prague Ruzyne Czechoslovakia NCIB = National Collection of Industrial Bacteria Aberdeen UK NCTC = National Collection of Type Cultures London UK

9 *Micrococcus* subgroup 1 according to Baird Parker (4)
10 *Micrococcus* subgroup 5 according to (4)
11 NCIB 2699 *Staphylococcus* sp classified in the type collection as belonging to *Staphylococcus* subgroup VI according to (4) Formerly designated *M freudenreichii* Corresponds to NCTC 2669 and to ATCC 8459
12 CCM 2392 *Staphylococcus salivarius*
13 CCM 2393 *S salivarius*
14 FDA 209p *S aureus* (= ATCC 6538 p NCTC 7447)

The following less related strains were subject to limited studies

15 CCM 537 *M morrhuae* (proposed neotype)
16 CCM 982 *M denitrificans* (= ATCC 13543 NCIB 8944 cotype)
17 NCTC 8251 *Aerococcus viridans*
18 C 6 *A catalasicus*
19 NCIB 6990 *Pediococcus acidilactici*
20 NCTC 8066 *P cerevisiae*

Strains 1–5 12 13 15 16 were obtained from Dr M Kocur Brno Czechoslovakia strains 7 and 8 were from Dr E Hamatova Czechoslovakia strains 9 and 10 were supplied by Dr A C Baird Parker England strain 14 came from Dr W A Zygmunt Evansville Ind U S A items 17–20 were from Dr O G Clausen Oslo Norway items 6 and 11 were obtained directly from the type collections Strains 13 and 14 were isolated and described by Gordon (19)

Sensitivity to antibiotics This was tested by a disc diffusion technique using paper discs technique and regression curves of the Bakteriologiska Laboratoriet Karolinska Sjukhuset Stockholm Sweden

Serological techniques The immune serum was made with strain 421/68 as antigen After centrifuging an overnight culture the cells were washed once in 0 5 per cent saline suspended in 25 ml phosphate buffer of pH = 7 4 ultrasonically disrupted treated at 70 C for 30 minutes and plated for sterility control before inoculation The rabbit was given 0 5 ml vaccine intravenously and 1 ml subcutaneously every 5th day Bleeding was performed after 2½ months when the titer had not risen during the previous 1½ months

The antigens for agglutination and capsular swelling reactions were overnight cultures treated at 100 C for 2½ hours and washed 3 times in physiological saline For tube agglutination 0 5 ml antigen was mixed with 0 5 ml diluted serum and incubated in a 37 waterbath for 18 hours For capsular swelling reaction antigens and serum dilutions were mixed on clean glass slides and examined microscopically in phase contrast

Lysostaphin susceptibility Lysostaphin was do-

nated by Dr W A Zygmunt of the Mead Johnson Research Center Evansville Ind U S.A and used according to his detailed written specifications (stencilled) and published technique (22 35)

Deoxyribonucleic acid (DNA) extraction DNA was extracted by a modification of *Marmur's* procedure (26) After growth at 37 C on 20 nutrient agar plates for 14 hours the bacteria were suspended in 40 ml citrated buffered saline (0 14 N NaCl + 0 015 M sodium citrate pH = 7 4) Then 200 μg/ml of lysozyme was added and the cells frozen at minus 20 C and thawed at 37 C for 30 minutes on three successive days

After adding 0 5 g sodium dodecyl sulphate the suspension was heated at 70 C for 10 minutes and subsequently at 56 C for another 10 minutes Later 8 ml 5 M sodium perchlorate and 40 ml of 4 per cent isoamyl alcohol in chloroform were added and the mixture shaken at 5 C for 20 minute The translucent supernatant which formed during centrifugation in an angle MSE high speed rotor at 10 000 rpm for 20 minutes was collected in a beaker DNA was then precipitated by 2 volumes of 96 per cent ethanol which was layered on top of the supernatant The grayish cloudy precipitate of DNA was wound up on a bent glass rod After draining off excess liquid the precipitate was dissolved in 3 ml citrate buffered saline

Further purification of DNA for determination of DNA base composition followed the procedure described by *Bovre et al* (12)

Determination of DNA base composition Analytical CsCl density gradient centrifugation was performed as reviewed by *Szybalski* (40) The mole per cent of guanine and cytosine (per cent (G + C)) was calculated from the buoyant density data using the simplified formula per cent (G + C) = 1000 (ρ–1 660) where ρ = buoyant density of DNA (g/cm³) measured versus the density of DNA from *Escherichia coli* assumed to be 1 710 g/cm³ and to contain 50 per cent (G + C)

RESULTS

Group A

The strains collected by us (Table 1) and two strains of *Staphylococcus salivarius* isolated by *Gordon* (19) CCM 2392 and CCM 2393

Microscopy All strains exhibited packets of coffee bean shaped Gram positive cocci of approximately 1 0–1 5 μ diameter There was a tendency towards diplococcal arrangement with the flattened sides next to each other All strains were Gram stable after three weeks in Heart Infusion Broth (Difco) A capsule was demonstrated by the method of *Møller* (29) The capsules appeared as unstained circular halos around the bacterial bodies The capsules were of PAS positive material (17)

Cultural characteristics The strains after overnight growth on blood agar exhibited 1–2 mm mucoid partly confluent transparent or greyish white colonies They had a shiny convex surface with regular borders and a gummy coherent consistency They were not easily suspendable and were partly adherent to the blood agar surface There was however, no sign of agar pitting After a few days the colonies would transcend from semitransparent to whitish opaque Colonies on Brain Heart Infusion Agar (Difco) were more easily suspendable There was a characteristic perhaps mouldy odour All strains were facultatively anaerobic (Table 2) None of the strains produced pigment A few displayed hemodigestion on human and bovine blood agar after several days at 4° C Strain 421/68 was particularly active in this respect but no haemolysis appeared No growth developed on coagulated serum slants or on bromothymol blue lactose nutrient agar plates In broth a viscid pellet formed Growth was augmented by aeration

Motility All strains were non motile in semisolid agar and in broth as observed microscopically

Biochemical reactions These are reported in Tables 2 and 3 The same preparation of soluble starch was used both in *Hugh & Leifson* medium where acid production was demonstrated by all strains and in the nutrient agar of *Baird Parker* (3) where no hydrolysis was revealed

Strains 5762/67 421/68 and MA 4399/69 hydrolyzed gelatin on *Clarke's* agar but did not split either arginine lysine or ornithine (30) The pH in litmus milk after 14 days ranged from 5 9 to 6 6 with a mean of 6 2 for six strains tested Skim Milk (Difco) turned slightly viscid but did not coagulate There was a separation of the curd but the precipitate did not form a solid clot A layer of clear whey was observed above the precipitate The same characteristics were observed with *Gordon's* two strains



Fig 1 Strain 2849/68 Methyl blue stain × 1300

Strains 5762/67 421/68, 623/68, 767/68 789/68 and 795/68 did not grow on *McLeod's* 0 4 per cent tellurite agar They grew on Heart Infusion Broth (Difco) agar plates with 20 per cent, but not with 40 per cent bile Other strains were not tested for these properties

Capsule formation An attempt to suppress capsule formation by growth in a minimal medium with amino acids added as growth factors failed The colonies were mucoid at all temperatures the width of the capsules varied with cultural conditions

Antibiotic sensitivity The strains were sensitive to ampicillin bacitracin chloramphenicol erythromycin fusidic acid lincomycin neomycin, novobiocin oleandomycin, oxytetracycline, and penicillin G All but three of the strains were sensitive to sulfonamide and all but one sensitive to streptomycin There was full or partial resistance to the following colistin gentamycin kanamycin, nalidixic acid and polymyxin B

Serological reactions All strains were agglutinated with the 421/68 serum titers ranging from 4 to 64 Capsular swelling was demonstrated in all but strain 795/68 Titers generally followed the agglutination titer and ranged from 2 till

Experimental at ty Five strain were tested for pathogenicity using 18 hours aerated Heart Infusion Broth (Difco) cultures No change was found in guinea pigs inoculated intraperitoneally with 0 5 ml of the culture Mice injected with 0 1–0 3 ml all developed abscesses upon subcutaneous injections These organisms were re isolated from the transparent viscid material in the abscesses One of the strains 421/68, produced a general effect in mice when an undiluted culture was injected subcutaneously, or intravenously The animals either died or developed a disturbance of balance within 2 days upon which the strain was isolated from the brain of the animals However a 1/100 dilution of a 421/68 culture did not elicit any changes after intraperitoneal intravenous or subcutaneous inoculations in four mice respectively

Changes in glucose bromocresol purple agar The medium designated by the Subcommittee on Taxonomy of Staphylococci and Micrococci for the differentiation of the two genera *Staphylococcus* and *Micrococcus* (39) showed a typical micrococcal reaction with our strains Acid production was only apparent in the upper 0 5–2 0 cm of the agar in the aerobic tube Distinctly less acid production evolved under anaerobic conditions

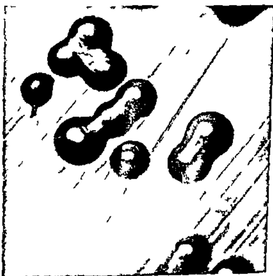


Fig 2 Colonies on human blood agar after over night growth × 10

TABLE 4 *Lysostaphin Susceptibility*

Strains	Mean per cent reduction of optical density (O D) 10 min	20 min	30 min	60 min
Group A*	7 0 (4 7–13 8)	8 3 (3 8–13 4)	11 3 (3 8–15 2)	13 3 (4 8–17 8)
M conglomeratus CCM 547	1 3	3 9	8 6	12 6
M luteus CCM 810	3 8	17 3	26 9	44 9
M roseus CCM 679	3 6	2 8	5 7	10 0
M sp Baird Parker s Subgroup 1	44 9	61 3	77 9	93 6
M sp Baird Parker s Subgroup 5	9 4	21 0	31 1	74 2
S aureus FDA 209 p	89 8	92 6	93 0	92 2

* Strains isolated by us (Table 1) CCM 2392 and CCM 2393

Lysostaphin susceptibility All strains were resistant to lysostaphin as seen in Table 4

DNA base composition The buoyant density of DNA extracted from the strains 2849/68 and 4239/68 was 1 719 g/cm³ corresponding to a per cent (G + C) content of 59 *Gordon* s strain CCM 2392 exhibited a DNA density of 1 720 g/cm³ equal to 60 per cent (G + C)

Group B

ATCC 407 and CCM 764 *Micrococcus freudenreichii* The two strains behaved identically

Microscopy The strains had Gram positive round cocci of about 1 μ diameter A capsule was demonstrated (29)

Cultural characteristics The strains exhibited 0 5 mm dome shaped colonies on blood agar after 18 hours After 2–3 days the colonies were 1–2 mm mucoid and partly confluent The colonies had shiny convex surfaces and their borders were regular The consistency was soft butyrous in contrast to the coherent consistency of the Group A strains The odour was weak uncharacteristic but distinct The colour was white after overnight incubation but changed to a yellow brown hue after a few days This change was observed on blood agar as well as on heart infusion plates The strains weres facultatively anaerobic No haemolysis was observed Growth was increased by aeration

Motility The strains were non motile

Biochemical reactions These are reported in Tables 2 and 5 The strains hydrolyzed gelatin on *Clarke* s plates (16) saponified olive oil by the method of *Bulder* (15) and failed to split arginine lysine or ornithine (30) Both Skim Milk (Difco) and Litmus Milk (Difco) separated with clear whey above the curd

Antibiotic activity The strains were sensitive to ampicillin bacitracin cephaloridin chloramphenicol erythromycin fusidic acid gentamycin kanamycin lincomycin neomycin nitrofurantoin oxytetracycline penicillin G polymyxin B streptomycin and sulfonamide They were resistant to colistin and nalidixic acid

Changes in glucose bromocresol purple agar The strains exhibited typical micrococcal reactions in the *Subcommittee* s medium (6 39)

DNA base composition Both strain CCM 764 and strain ATCC 407 were assayed and their DNA found to have a buoyant density of 1 718 g/cm³ corresponding to 58 per cent (G + C)

Group C

CRIPP A6 and CRIPP A171 *M freudenreichii*

Microscopy Gram positive round cocci of about 1 μ were found No capsule was demonstrated

Cultural characteristics After overnight growth on blood agar the strains showed white colonies o 0 5 d ameter They had entire edges A t ad some tend

TABLE 5 *Acid Production from Alcohols and Carbohydrates of Group B C and D Strains**

	Group B	Group C	Group D
Monosaccharides			
Pentoses			
l Arabinose	—	+	—
Rhamnose	—	—	+
d Xylose	—	+	—
Hexoses			
d Fructose	—	+	+
d Galactose	—	—	—
d Glucose	—	+	+
d Mannose	—	+	+
Disaccharides			
Cellobiose	—	—	—
d Lactose	—	+	+
Melibiose	—	—	—
Maltose	+	+	+
Saccharose	—	+	+
Trehalose	—	+	—
Polysaccharides			
Inulin	—	—	—
Raffinose	—	†	—
Starch	—	—	+
Alcohols			
Adonitol	—	+	—
Dulcitol	—	—	—
Inositol	—	—	—
Mannitol	—	+	+
Sorbitol	—	+	(+)§
Glycerol	—	+	+
Glucoside			
Salicine	—	—	(+)

* Group B = ATCC 407 and CCM 764 Group C = CRIPP A 6 and CRIPP A 171 Group D = NCIB 2699

† A 6 positive A 171 negative

§ (+) indicates a faint colour change insufficient to be clearly indicative of a positive reaction

ards dentate edges after some days growth The colonies were not mucoid and in appearance very similar to *Staphylococcus epidermidis* Their consistency was soft and friable They had a rancid penetrating odour The strains were non haemolytic and facultatively anaerobic

Motility The strains were non motile

Biochemical reactions These are recorded in Tables 2 and 5 Strain A 171 changed the milk slightly but did not coagulate it A typical micrococcal pattern evolved in the *Subcommittee* s glucose bromcresol purple medium (6, 39)

Antibiotic activity Sensitivity to antibiotics was the same as in the Group B except that the Group C strains also were sensitive to colistin and resistant to bacitracin

Group D

NCIB 2699 *Staphylococcus* subgroup VI (3 4) This strain had formerly been named *M freudenreichii*

Microscopy The strain had Gram positive round cocci but no capsule (29) Chains of cells were not observed after growth in broth

Cultural characteristics Overnight growth on blood agar showed 0 2–0 4 mm slightly opaque colonies resembling group A streptococci The colonies were shiny convex but not mucoid and had entire edges No haemolysis appeared A faint odour was discernible Good growth occurred at room temperature at 47° C and anaerobically at 37° C

Motility The strain was non motile

Biochemical reactions These are recorded in Tables 2 and 5 A solid clot formed in Skim Milk (Difco) A typical staphylococcal pattern developed in the *Subcommittee* s medium (6, 39)

Antibiotic sensitivity The strain was sensitive to ampicillin cephalosporidin chloramphenicol erythromycin fusidic acid lincomycin neomycin nitrofurantoin oxytetracycline penicillin G and streptomycin It was resistant to bacitracin colistin gentamycin kanamycin polymyxin B and sulfonamide

Group E

Strains not reported above (see methodological section)

Either by complete or partial examination pronounced differences were found between these strains and the Group A strains The *S aureus* FDA 209 p was lysostaphin susceptible whereas the degree of lysis varied with the micrococci (Table 4) The other data will not be reported in detail here the Group A strains were sufficiently different to be grouped as a separate species However

for method evaluation purposes it may be mentioned that *Aerococcus viridans* was catalase negative and *A catalasicus* very weakly positive

DISCUSSION

As seen from Tables 2 3 and 4 the Group A strains form a homogeneous group However only two strains (5762/67 and 421/68) showed catalase reactions of intensity comparable to *M luteus* The change observed in skim milk *i e* an increase in viscosity and separation of the milk without any *bona fide* coagulation was typical of all these strains No such change was observed in litmus milk The susceptibility to changes in environmental conditions particularly their low salt tolerance set them apart from many other micrococci The species is readily recognizable by colonial appearance consistency and in particular its adherence to blood agar surface It is noteworthy that none of the pentoses and only one of the alcohols were acidified It should be noted that acidification was rather weak with all Group A strains Our serological data confirm the homogeneousness of this group all strains reacting with the same serum

Included in Group A are two strains isolated by *Gordon* (19) which were of the same species as our strains *Gordon* reported that nitrate was reduced beyond nitrite We found that nitrite accumulated with all strains and that gas production was not discernible with *Durham* tubes *Gordon*'s findings that his strains liquefied 4 per cent but not 12 per cent gelatin is of considerable importance since this is in accord with our finding that these strains are slow gelatin liquefiers A longer observation period (14 days) however reveals that even 15 per cent gelatin is liquefied Still there is no doubt that our strains in Group A belong to the entity *Staphylococcus salivarius* Andrewes & Gordon 1907 (1) and that *Gordon* (19) studied the same entity

By virtue of their DNA base composition the Group A strains must belong to the genus *Micrococcus* The Group A strains have a per cent (G + C) of 59–60 Using thermal denaturation *Gordon* found his strains to vary from 55 4 to 57 3 per cent (G + C) His strain CCM 2393 was found by us to have 60 per cent (G + C) A compilation of all available per cent (G + C) determinations for the *Micrococcaceae* shows a continuum of values from 54 2 (*M varians*) (33) to 75 0 (*M luteus*) per cent (G + C) (10) This interval includes the type species for the genus *Micrococcus M luteus* and therefore must correspond to this genus In comparison the range of values corresponding to the genus *Staphylococcus* is 30 0 (*S candidus*) (2) to 38 8 (*M candidans*) per cent (G + C) (36) Between these two intervals are the values of some Gram positive motile cocci referable to the genus *Planococcus* (10 11 29) The range for these strains so far is 39 6–51 2 (10 11)

Thus DNA base composition is a criterion of fundamental value in the distinction between staphylococci and micrococci Other criteria suggested for the separation of these genera are glycolysis (39) and lysostaphin resistance (22 35 36) In these respects also the Group A strains behaved as typical micrococci Notwithstanding this the glucose bromocresol purple method (39) has definite limitations (24 31)

Consequently the Group A strains belong to the genus *Micrococcus* Due to this fact the classification given to them by *Gordon* (19) *Staphylococcus salivarius* (1) would have to be changed (8) However it appears that an earlier name is available for this species *M mucilaginosus* Migula 1900 (27) Their coffee bean shape diplococcal arrangement size their behaviour in milk and low gelatinase activity all support this *Krasilnikov* (25) gives further foundation to this by stating tha this species reduces nitrate to nitrite and do not grow with ammonium compounds as s source of nitrogen

Still the milarity between *M mucilaginosus* and oth r taxa remains of interest The weak catalase production of the Group A strains makes it conceivable that they could erroneously have been referr d to an entirely

TABLE 5 *Acid Production from Alcohols and Carbohydrates of Group B C and D Strains**

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Monosaccharides			
Pentoses			
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Rhamnose	—	—	+
d Xylose	—	+	—
Hexoses			
d Fructose	—	+	+
d Galactose	—	—	—
d Glucose	—	+	+
d Mannose	—	+	+
Disaccharides			
Cellobiose	—	—	—
d Lactose	—	+	+
Melibiose	—	—	—
Maltose	+	+	+
Saccharose	—	+	+
Trehalos	—	+	—
Polysaccharides			
Inulin	—	—	—
Raffinose	—	†	—
Starch	—	—	+
Alcohols			
Adonitol	—	+	—
Dulcitol	—	—	—
Inositol	—	—	—
Mannitol	—	+	+
Sorbitol	—	+	(+)§
Glycerol	—	+	+
Glucoside			
Salicine	—	—	(+)

* Group B = ATCC 407 and CCM 764 Group C = CRIPP A 6 and CRIPP A 171 Group D = NCIB 2699

† A 6 positive A 171 negative

§ (+) indicates a faint colour change insufficient to be clearly indicative of a positive reaction

wards dentate edges after some days growth The colonies were not mucoid and in appearance very similar to *Staphylococcus epidermidis* Their consistency was soft and friable They had a rancid penetrating odour The strains were non haemolytic and facultatively anaerobic

Motility The strains were non motile

Biochemical reactions These are recorded in Tables 2 and 5 Strain A 171 changed the milk slightly but did not coagulate it A typical micrococcal pattern evolved in the *Subcommittee*'s glucose bromcresol purple medium (6 39)

Antibiotic activity Sensitivity to antibiotics was the same as in the Group B except that the Group C strains also were sensitive to colistin and resistant to bacitracin

Group D

NCIB 2699 *Staphylococcus* subgroup VI (3 4) This strain had formerly been named *M freudenreichii*

Microscopy The strain had Gram positive round cocci, but no capsule (29) Chains of cells were not observed after growth in broth

Cultural characteristics Overnight growth on blood agar showed 0 2–0 4 mm slightly opaque colonies resembling group A streptococci The colonies were shiny convex but not mucoid and had entire edges No haemolysis appeared A faint odour was discernible Good growth occurred at room temperature, at 47° C and anaerobically at 37° C

Motility The strain was non motile

Biochemical reactions These are recorded in Tables 2 and 5 A solid clot formed in Skim Milk (Difco) A typical staphylococcal pattern developed in the *Subcommittee*'s medium (6 39)

Antibiotic sensitivity The strain was sensitive to ampicillin cephalosporidin chloramphenicol erythromycin fusidic acid, lincomycin neomycin nitrofurantoin oxytetracycline penicillin G, and streptomycin It was resistant to bacitracin colistin, gentamycin kanamycin polymyxin B and sulfonamide

Group F

Strains not reported above (see methodological section)

Either by complete or partial examination pronounced differences were found between these strains and the Group A strains The *S aureus* FDA 209 p was lysostaphin susceptible, whereas the degree of lysis varied with the micrococci (Table 4) The other data will not be reported in detail here the Group A strains were sufficiently different to be grouped as a separate species However

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TABLE I *The reaction of T S and M R red cells in different testsera*

	T S red cells Reaction	M R red cells Reaction
in anti B	+*	+ + +*
anti A	—	—
anti D	+ +	+ + +
anti C	+ +	+ +
anti E	+*	+ + +*
anti e	+ + +	+ + +
anti c	+ +	+ + +
anti M	+ + +	+ + +
anti N	+ +	+ + +
anti S	+ +*	+ + +*
anti s	+ + + +	+ +
anti P	+ + +	+ + +
anti Le	—	—
anti Fya	+ + +	+ + +
anti K	—	—

d cells is added 0 25 ml of this suspension is ixed with equal parts of selected strong anti B id AB serum respectively in 2 Widal tubes Af r incubation of the mixture for half an hour at) C it is centrifuged for 1 minute at 500 G id the tubes are tapped sharply to break up the umps of red cells from the bottom This is re eated once After another centrifugation the tu es are turned upside down 50 times and left for ne minute for sedimentation By means of a capil ry tube both chambers of a Burker Turk count ig chamber are filled with the supernatant An rea corresponding to approx 1000 cells from the ontrol tube is counted

Result of differential agglutination study

T S 73 per cent O red cells and 27 per cent 3 red cells

M R 31 per cent O red cells and 69 per cent 3 red cells

Control on the technique was made with known mixtures of B and O red cells

Study of the red cells of the parents of T S and M R has not been made as this in the present case has no actuality bloodchimerism not beeing hereditary

DISCUSSION

Human blood group chimerism is usually de tected by irregularities in the ABO grouping either in the form of weak reactions possibly associated with mixed field agglutination in testing the red cells in anti A or anti B test sera or in the form of lacking anti A or anti B in the serum at the serum control This makes it difficult to distinguish blood group chim erism serologically from weak variants of A or B

The diagnosis may be established by study ing a blood sample from the other twin whose red cells will then give a similar pattern of agglutination However, the other twin may have died perhaps already in utero (*Duns ford* 1953) In that case the diagnosis has to be based on other criteria e g mixed field agglutination within other blood group sys tems, in the present case E and S, or a study of leucocytes or platelets as suggested by *Mia van der Hart* (1967)

Absence of A or B substance in the saliva of normal secretors of H substance associated with weak agglutination and mixed field ag glutination of the red cells in anti A or anti B may also be helpful in diagnosing chimerism, the secretion of A or B-substance taking place only in a zygote who from the moment of conception possesses the A or B gene plus the secretor gene Thus T S has approx 30 per cent B red cells in his circulating blood but does not secrete B substance Since he is secreting normal quantities of H substance this means that he has the B gene only in the primordial cells of the red group B cells not in other cells in his body specially not in the mucin producing cells in the salivary glands

The present case of blood group chimerism includes the antigens O and B besides E and S on the red cells Weak B variants are ex tremely rare in the European population Therefore the possibility of chimerism should be borne in mind in cases where a weak or absent reaction of the red cells in anti B test serum is associated with absence of anti B in the serum at the serum grouping The im plantation of even relatively few A or B primordial red cells into an individual lacking the A or B gene will often result in lack of production of the normal serum agglutinin corresponding to the implanted cells if the implantation takes place at an early stage of foetal life However this immunological tole rance is not h / te as it has been demon

strated by Dunsford et al that the tolerance may be temporarily broken down during pregnancy (*Dunsford* 1957)

In man sterility has not been found as a concomitant phenomenon in blood group chimerism Mrs M R had a history of a normal pregnancy

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STUDIES ON THE COMPLEMENT FIXATION TEST WITH *MYCOPLASMA PNEUMONIAE* ANTIGEN

5 Time Pattern of Not Cell Bound Antigen in Broth Culture

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The time pattern for the early development of not cell bound complement fixing antigen (NCBA) of *M pneumoniae* Bard strain in filtrates from broth cultures has been examined A filter disc technique for the preparation of washed cell inoculates which are free of NCBA and have good cell viability is described Using this technique NCBA could be demonstrated significantly earlier than the culmination of the number of CFU/ml i e during the late growth phase of the cultures No NCBA could be traced during the first days of incubation The precipitability of NCBA with ammonium sulphate is reported and a preliminary precipitation technique has been employed in the demonstration of NCBA together with the direct measurement technique reported earlier (3 4) The sensitivity of the former method proved to be higher than that of the latter one It is also reported that growth of *M pneumoniae* colonies can be initiated from Millipore® 0 22 μ filter discs that have been used for filtration of broth cultures of the organism It is suggested that the significance of this fact in the isolation of *M pneumoniae* from fluid materials should be investigated

The antigenic properties of *M pneumoniae* have been studied by a number of authors (7 8 9 10 see also references cited in (3)) The usual starting material has been *M pneumoniae cells* obtained usually by centrifugation of broth cultures or by scraping off *M pneumoniae* growing on a glass surface (11) The existence in broth cultures of complement fixing antigen which is non centrifugable at abt 30 000 G has been noted by *Somerson et al* (11) using chloroform methanol extraction *ad modum Kenny & Grayston* (6) and by *Eng* (3) using a direct measurement of the complement fixing (CF) titre in the inactivated supernatant By means of the latter technique *Eng* (4) further demonstrated CF antigen in broth culture filtrates which were free of viable cells In the present study the development of not cell bound CF antigen (NCBA) in broth cultures of *M pneumoniae* has been further examined in relation to the growth curve of the cultures A simple and rapid filter disc method is described for the preparation of washed cell inoculates which are free of NCBA and have good cell viability It was found during the present study that NCBA can be precipitated from the filtrates by means of ammonium sulphate and this has been employed as a method of measuring NCBA with a sensitivity higher than that of the direct measurement

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TABLE 1 *The Early Development of Not Cell Bound Complement Fixing Antig n in Broth Cultures of* M pneumoniae *Bard Strain in Relation to the Growth Curve in 3 Experiments*

Exp	No of CFU/ml at day 0	Highest No of CFU/ml measured	Time of culmination of No of CFU/ml	CF titres		
				at day	in filtrate measured directly	in (NH) SO_4 precipitate from filtrate
1	2 10^4	2 10^6 at day 8	Later than day 8	0–4	—	—
				5	—	1 2
				6	1 2	1 8
				7	1 4	1 16
				8	1 32	1 256
2	2 10^3	5 10^6 at day 10	Later than day 10	0–8	—	—
				9	—	1 2
				10	1 4	1 32
3	8 10^4	7 10^6 at days 6 and 7	*Days 6 and 7*	0–3	—	—
				4	—	1 2
				5	1 1	1 4
				6	1 4	1 16
				7–9	1 4	1 16

CFU = colony forming units

M pneumoniae cells from broth cultures is also reported

MATERIAL AND METHODS

The cultivation of *M pneumoniae* Bård strain and the production of rabbit antiserum are described in (2) Counting of the number of colony forming units (CFU) in broth culture was done by titration of the culture in PPLO broth in $\log_{10}$ dilution steps From each dilution 0 05 ml was immediately spread on a PPLO agar plate and the colonies were counted after 10 days incubation by means of the haemolytic plaque technique (for details and references see (3)) —Broth cultures were filtered through Millipore® filters (MF) type GS (stated pore diameter 0 22 μ) with a diameter of 47 mm under reduced pressure by suction —The CF titres were measured directly in the filtrates after inac tivation at 56 C for 30 min as described by *Eng* (3 4)

A washed cell inoculum to start growth of *M pneumoniae* in broth culture was prepared as fol lows An ordinary broth culture was made as de cribed in (2) with finely cut agar culture a the inoculum After 3 days incubation the agar r asses were removed by filtration through sterile gauze Ten ml of the culture was tl n filtered through an autoclaved MF 0 22 μ filter disc Af er the filtration was completed tl e filter disc ›s washe l 3 times each tim by passi ıg 10 ml of l› broth medium through the filter The filter disc was then trans ferred aseptically to a flask containing 1000 ml broth medium serving as inoculum in the experi mental broth culture —Preliminary experiments on this preparation technique showed

CF titre in broth culture	1 16
CF titre in filtrate	1 16
CF titre in first washing	1 4
CF titre in second washing	1 1
CF titre in third washing	neg

Precipitation of NCBA with ammonium sul phate To 32 ml filtrate was added 7 0 g $(NH_4)_2 SO_4$ *pro analysis under continuous stirring which* was continued at +4 C for 30 min The mixture was then centrifuged at 15 000 rpm for 10 min The centrifugate was resuspended in 2 ml 0 9 per cent NaCl dialysed twice for several hours against 200 vol saline inactivated at 56 C for 30 min and titrated for measurement of CF titre

EXPERIMENTAL

Broth cultures of *M pneumoniae* Bård strain were made up with washed cell inoculates as described The following examinations were performed daily starting with day 0

1 The number of *CFU/ml broth culture* was measured Four dilution steps were generally plated from each titration row and the number of CFU/ml generally calculated from plates sho ıng 10 100 haemolytic plaques

2 About 40 ml of the broth culture was filtered through MF 0 22 μ The existence of *M pneumoniae* cells in the filtrate was examined

a by spreading 0 05 ml of the filtrate on a PPLO agar plate b by adding 0 2 ml of the filtrate to a tube containing 1 8 ml PPLO broth medium with 1 per cent glucose and 0 002 per cent phenol red added (references are given in (4)) The tubes were incubated for 3 weeks Growth of *M pneumoniae* in the tubes was indicated by a change in colour from red to lemon yellow

3 The CF titre in the filtrate was measured

4 The filtrate was precipitated with (NH_4) SO_4 and the CF titre in the precipitate was measured

RESULTS

The early development of NCBA in relation to the growth curve in three experiments is presented in Table 1 NCBA was not found in filtrates from broth cultures during the first days of incubation The point of time for the first demonstration of NCBA was significantly earlier than the culmination of the growth curve in two of the three experiments performed The NCBA titre increased during the days following the first detection NCBA was demonstrated one day earlier in the (NH_4) SO_4 precipitates than in the filtrates measured directly in all experiments Twelve complement fixing precipitates were harvested altogether The CF titres in two of these were 3 log dil steps and in the remaining ten precipitates 2 log dil steps higher than those in the corresponding directly measured filtrates The highest CF titre measured directly in a filtrate was 1 32 and in a precipitate 1 256—Uninoculated control broth medium and (NH_4) SO_4 precipitate from the medium did not fix complement in the presence of anti serum against *M pneumoniae*—The results obtained in Exp 3 are shown diagrammatically in Fig 1

In Exp 1 the CF titres in the unfiltered broth cultures were also measured directly they were less than 1 log dil step higher than in the corresponding filtrates

During the first part of this work the MF filter disc was renewed during the filtration procedure using the same filter base The resulting filtrates showed from < 2 10^1 to

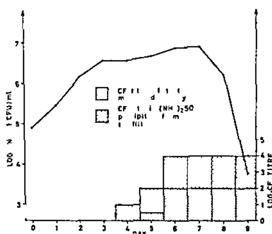


Fig 1 The early development of not cell bound complement fixing antigen in broth culture of *M pneumoniae* Bard strain in relation to the growth curve (exp 3) CFU = colony forming units

8 10^1 CFU/ml Later on, the whole volume of broth culture was filtered through one filter disc or two filtration sets were employed Altogether fifteen broth cultures were filtered under the latter conditions Table 2 shows the number of CFU/ml in these filtrates and the corresponding broth cultures Ten filtrates gave no colonies (< 20 CFU/ml) whereas 5 filtrates contained from 8 10^1 to 2 10^3 CFU/ml The broth tubes inoculated with the former 10 filtrates did not show a change in colour whereas all the tubes containing the latter 5 filtrates changed from red to

TABLE 2 *Efficiency of Millipore® Filters Type GS (0 22 μ) in Removing Colony Forming Units from Broth Cultures of* M pneumoniae *Bård Strain The Correlation between No of CFU/ml in 15 Broth Cultures and the Corresponding Filtrates is Shown*

No of CFU/ml in filtrate	No of CFU/ml in broth culture				
	10^3–10^4	10^4–10^5	10 – 10^6	10 – 10^7	Total
< 2 10^1	1	1	2	6	10
10^1–10				1	1
10 –10^3				3	3
10^3–10^4				1	1
Total	1	1	2	11	15

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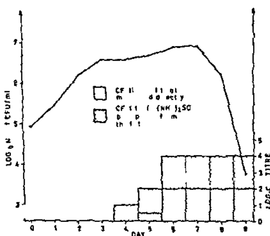


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corresponding to the Fab line while a long precipitation line was formed with γG globulin incubated without enzyme

All the pepsin digested γG globulins were inactive in agar gel diffusion against protein A Neither did the pepsin digested preparations inhibit the precipitation of protein A by normal human serum In contrast to this the precipitation of protein B by γG globulin from immune sera was inhibited by pepsin digested immune γG globulin (Table 1) These results suggest that protein A combines with the Fc region of the γG globulin molecule whereas protein B combines with the Fab region

Pepsin digested γG globulin from all sera tested retained its ability to agglutinate Cowan I bacteria, but did not agglutinate TSE sensitized with crude protein A On the other hand pepsin digested γG globulins blocked the sensitizing substance of crude protein A preventing its inhibitory effect (Table 2 and 3) It may therefore be concluded that both in bacterial agglutination and indirect haemagglutination the active sites of the γG globulins are at the Fab region

DISCUSSION

γG globulins precipitating protein A seem to occur frequently in normal sera All sera from 6 of 14 species gave the protein A line on agar gel diffusion The present findings that protein A reacts with γG globulins H chains papain digested but not pepsin digested γG globulins are in agreement with those of *Forsgren & Sjoquist* (3 4) and *Forsgren* (1 2) They concluded that the reaction between γG globulin and protein A is not a true antigen antibody reaction but is non specific and that a part of the γG globulin molecule other than the active site of the acquired antibody is involved Their findings and conclusion are supported by the present results with the exception that normal rabbit γG globulin showed no activity with protein A In all normal sera and immune sera precipitating protein A this reaction seem to involve the Fc part of the γG globulin molecule

The finding that protein A seems to combine with the same region of normal and immune γG globulins might indicate that this ability of normal γG globulin is induced by *Staph aureus* or other bacteria containing protein A or cross reacting antigens This is however not likely Protein A or cross reacting antigens were not detected in the 13 bacterial species tested We assume that in contrast to polar bears our rabbits must frequently be in contact with coagulase positive staphylococci However normal sera from polar bears precipitated protein A in relatively high titres whereas normal rabbit sera or isolated γG globulin did not Furthermore, sera from all individuals within each species tested were either positive or negative with respect to precipitation of protein A

The disagreement between our results and those of *Forsgren & Sjoquist* (4) concerning the activity of normal rabbit γG globulin is interesting It may indicate that normal γG globulin from some breeds has an ability to precipitate protein A which is not present in others

The Fc region of the γG globulin is the site of attachment of complement Protein A and sera do not fix complement (5) This may indicate that protein A, when combining with Fc, does not induce the structural changes of the γG globulin necessary for complement fixation or that complement fixation is sterically hindered by protein A

None of the normal sera precipitated protein B and this antigen combined only with the Fab fragments of immune γG globulins indicating a true antigen antibody reaction

Almost all normal sera tested agglutinated *Staph aureus* Cowan I bacteria Exceptions were sera from 1 rabbit 1 rat and 2 mice Since the mouse sera precipitated protein A, and no correlation between the titres of agglutination and precipitation reactions was observed any connection between these reactions is improbable Furthermore the pepsin digested γG globulins still agglutinated the bacteria indicating a normal antigen anti

body reaction Similarly the active site or ability of the γG globulins to agglutinate TSE sensitized with crude protein A was localized to the Fab region

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only quantitatively different from those found in immune mice This speaks in favour of the contention that the protective factors present both in immune and conventional mice might be of a similar nature

The results show that SPF animals may be useful tools in the study of certain host parasite relationships

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This study was supported by the Danish State Research Foundation (Statens almindelige Videnskabsfond)

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THE FATAL HAEMORRHAGIC DISEASE OF CHICKEN EMBRYOS INJECTED WITH NORMAL ALLANTOIC FLUID

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The already known toxicity of normal chicken allantoic fluid (AF) for the embryo itself or for other chicken embryos has been analysed and found to be due the content of thromboplastic lipo proteins in the AF The haemorrhagic syndrome caused by i v injection of the embryo with AF is suggested as an experimental model for the study of intravascular clotting and the defibrination syndrome The lethality of the haemorrhagic syndrome could be largely prevented not only by heparin but also by natural inhibitors present in both chicken serum and a variety of mammalian sera At least one of these inhibitors has been isolated from mammalian sera and identified as the previously described antithrombin III (progressive antithrombin)

Virologists seem to have known for long that the allantoic fluid of chicken embryos is toxic Thus in their manual on the use of chicken embryos in virological research when *Beveridge and Burnet* (1946) recommended a standard volume of inoculation of 0 05 ml for i v injection of virus containing material they also warned their fellow virologists that However as little as 0 01 ml of normal allantoic fluid kills the embryo in a few minutes with multiple haemorrhages and some foreign tissue extracts have a similar action

I have in principle observed a similar toxicity of normal allantoic fluid although the lethal dose is in my hands about 10 times higher and even then death requires 1–2 hours rather than a few minutes Preliminary tests soon showed that the toxic principle was a high molecular substance which did not pass the dialysis membrane Thus the toxicity was not due to simple metabolic waste products accumulated in the embryonic bladder the allantoic sac

Curiosity prompted the present inquiry into the nature of this apparent auto toxicity which seemed unexplained

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MATERIALS AND METHODS

Chicken Embryos

The vast majority of the embryos used happened to be a first generation cross between Rhode Island Red and White Leghorns but there was no indication that the breed of chickens mattered

Allantoic Fluid (AF)

The AF was harvested from embryos of different ages most often at the 17th day of incubation The egg shell was broken over the air sac and the underlying shell membrane lifted off with fine forceps This usually caused some oozing of blood from chorio ic vessels which was allowed to stop spontaneously before the chorio allantoic membrane was opened and th AF removed by Pasteur pipettes The fluids were kept individually

only quantitatively different from those found in immune mice This speaks in favour of the contention that the protective factors present both in immune and conventional mice might be of a similar nature

The results show that SPF animals may be useful tools in the study of certain host parasite relationships

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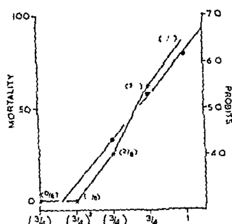


Fig 3 Titration of the toxicity of allantoic fluid Horizontal axis relative concentrations of AF Crude mortality data (x), and probit transformed data (●)

dilution step Therefore 2 fold dilutions will often yield a single dilution step in the mid lethal range while the neighbouring concentrations kill either all or none

Fig 3 shows a titration with more closely spaced doses (a 4/3 fold dilution series) As to be expected this gives an approximation to a sigmoid curve which is straightened considerably by probit transformation The empirical probits shown in Fig 3 give a regression of 7 3 per logarithmic cycle (i e per ten fold dilution) This happens to be the same as the mean regression found in 11 independent experiments where AF harvested from 13 to 18 day old embryos were injected into embryos aged from the 11th day to the day of hatching (21 st) There was no systematic variation of the regression with either donor or host age while the LD 50 as we shall see later, varies with both

Survivors from a sublethal injection of AF, if examined a few hours later, may show the same haemorrhagic lesions as seen in the dead ones However, the lesions are on the whole more widespread in those which actually succumb If survivors are examined one day later, it is still possible to see remnants of the characteristic muscular petecchiae, but resorption and repair is obviously well advanced

It was observed that a few survivors from a midlethal dose which were later injected with an LD 100 still failed to die This observation was repeated more systematically in the experiment shown in Table 1 It appears that there is indeed a protective effect of a sublethal dose and that protection requires more than 30 seconds to take effect but is fully developed in 20 minutes Furthermore that the protection lasts only for a few hours and then fades away gradually

It was tried without success to transfer this kind of protection passively with transfusion of citrated whole blood from protected to normal embryos prior to injecting the latter with an LD 100

TABLE 1 *Protection against a Lethal Dose of Allantoic Toxin from Prior Injection of a Sub Lethal Dose of the Same Preparation*

Dose of 1st injection	Interval	Dose of 2nd injection	Mortality at 2 hours after last injection (no dead/no inject)
/3 Unit*	–	None	1/20 (LD 5)
–	20–30 sec	1 Unit*	9/10
–	20 min	–	0/10
–	90 min		0/10
–	4 hours	–	5/10
None	–	–	20/22 (LD 90)

* The unit is chosen arbitrarily as 0 1 ml of the undiluted preparation which in this experiment was an AF pool from 50 17 day White Leghorn embryos The fluid was dialysed twice at 4 C for 9 hours each against phosphate buffered saline of pH 7 35 All injections were given i v as 0 10 ml volume



Fig 1 Typical haemorrhagic lesions in the muscles and meninges of a 13 day embryo dying from i v injection of allantoic fluid

until it had been ascertained that the amniotic sac was intact and were then pooled The pooled AF was dialysed first against tap water to remove urates and other dialysable substances and then against isotonic phosphate buffered saline (PBS) at pH 7 3 The small remaining sediment was removed in a bench centrifuge The supernatant fluid which carried the full toxicity of the native AF was stored at − 20 in test tubes containing about 3 ml each so as to avoid re freezing of tubes which had been thawed already

The toxic principle was found to be sedimentable almost completely by spinning for 1 hour at 50 000 rpm Resuspensions of such sediment were used in some experiments instead of the native AF It was found to consist of approximately 30 to 40 per cent protein 50 to 60 per cent lipids and 2 to 5 per cent RNA

The Embryonic Test System

A window is made in the egg shell of a 13–14 day embryo over the site of a chorio allantoic vein and AF is injected with a 30 gauge needle mounted to a tuberculin syringe

A few minutes later trans illumination of the (preferably white) egg reveals that the embryo moves more sluggishly than before The chorio allantoic arteries (which carry the de oxygenated blood to the membrane) become markedly darker in colour, and a general pallor develops of areas between major vessels No similar changes occur from control injections of saline or chicken serum

With the above mentioned dose injected into 13–14 day embryos some may recover but the majority die within 1–2 hours Almost all 2 hour survivors will also be alive the next day Hence survival rate at 2 hour post injection has been used in this study as a convenient end point of analysis

Post mortem examination shows in some cases a large haematoma at the site of injection, quite unlike the tiny blood spot left by control injections of saline In such cases the embryo itself shows of course a deep anaemia

Much more common, however, is the finding of a small haematoma at the injection site coupled with petecchiae in the skin sceletal muscles myocardium, lungs, and stomach wall (Fig 1–2) Surprisingly few are seen in liver kidneys and brain and none in the gonads and the bursa of Fabricius Bleeding is frequent into the serous body cavities

EXPERIMENTAL ANALYSIS

Dose Dependance and Protection from Prior Sublethal Dosage

The toxic agent shows by i v injection a dose dependance with rather a steep slope If 2 fold dilutions are made, most of the range from zero to 100 per cent mortality is for a given preparation contained in a single



Fig 2 Hearts of 17 day embryos *Left* heart from an embryo injected with a lethal dose of allantoic fluid *Right* heart from a control injection with same volume of PBS

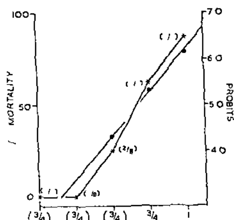


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TABLE 2 *Mortality from Injection of the Same Pool of Dialysed 17 Day Allantoic Fluid into Chick Embryos of Different Ages*

Recipient age (days)	Injected dose*	Deaths/injected 2 hours later	Approximate LD 50†	Body weight (g)	$\frac{LD\ 50 \times 100}{\text{Body weight}}$
10	0 032	5/9			
10	0 040	5/8			
10	0 050	7/8	0 034	2 5	1 4 ml
13	0 025	0/5			
13	0 050	0/5			
13	0 100	11/21			
13	0 200	5/5	0 097	7 5	1 3 ml
17	0 200	0/12			
17	0 300	3/12			
17	0 400	7/8	0 320	20 0	1 6 ml
21	0 7	0/3			
(newly§	1 0	2/5			
hatched)	1 5	4/5	1 100	37 0	3 0 ml

* Dose expressed in ml of undiluted AF although the inoculated volume was never less than 0 10 ml (dilutions were made in isotonic phosphate buffered saline of pH 7 3)

† LD 50 estimated from plotting of the probit transformed data on graph paper on assumption of a common slope of 7 3

§ 1 5 ml of phosphate buffered saline caused no lethality in 5 chicks

TABLE 3 *Organ Distribution of Primary Haemorrhagic Lesions* in Embryos of Different Age Receiving a Lethal Dose*

Age (days)	Skin	Muscles	Heart	Lungs	Stomach	Spleen	Thymus	Meninges†
10	+ +	+	0	0	0	0	0	+ +
13	+ +	+ +	+	+ +	+	0	0	+
17	0	+ +	+ + +	0	+	+	0	+ +
21 (hatched)	0	0	0 but clots inside	0	0	0	+	(+)

* Severe stasis was seen sometimes in lungs liver and kidneys often associated with extravasation of blood in the pleurae and the peritoneum Though often very spectacular in 13 and 17 day embryos these changes are probably secondary to heart lesions and are not tabulated in Table 3

† Although meningeal bleeding was very frequent there were hardly ever lesions found in the brain itself.

Changes in Susceptibility and Pathology with Embryonic Age

Table 2 summarizes mortality data from injection of the same dialysed 17 day AF preparation into embryos of different stages of development It is apparent that in the range from 10–17 days of incubation there is very little difference if any, in the body weight adjusted dose which represents an LD 50

In spi e of this fact ie w s a difference to be seen in the distribution of haemorrhagic lesions (Table 3) The most spectacular differences concerned the heart and the skin The 17 day embryos showed practically none of the petecchial bleeding in the skin which is so typical of the younger embryos On the other hand the heart was regularly much more severely damaged in the older embryos

The gross appearance of the injected 17 day embryo heart resembled sometimes total haemorrhagic infarction (Fig 2) Micro-

TABLE 4 *Relative Toxicity of 5 Pools of Allantoic or Amniotic Fluid Injected I I into 13 Day Embryos*

Injected fluid	Dose	Deaths/ Injected	LD 50* (ml)	µg protein† per ml	µg protein per LD 50
AF 10 day	0 50	1/9	0 68	375	255
PBS	0 50	0/9	–	–	–
AF 13 day	0 40	7/9	0 33	425	140
– –	0 30	3/9	–	–	–
AF 17 day	0 12	9/9	0 076	625	47
– –	0 08	5/9	–	–	–
AF 18 day	0 060	12/14	0 048	925	45
– –	0 045	2/7	–	–	–
AMF 18 day	0 20	5/7	?	80 000	–
– –	0 10	6/7	–	–	–
– –	0 05	4/7	–	–	–
Same retested	0 30	8/8	appr 0 05	–	appr 4 000
5 weeks later	0 15	8/8	–	–	–
*	0 075	8/8	–	–	–
	0 038	0/8	–	–	–
	0 019	0/8	–	–	–

* LD 50 estimated as in Table 2

† *Protein estimated by Lowry s method*

§ AMF had been kept at —20 C betv een the 2 assays but had been briefly thawed in between which procedure produced a slight insoluble precipitate

scopically there was particularly massive bleeding in the pericardium but also the myocardium was infiltrated with blood from distended and in places ruptured capillaries Large clots were seen adhering to the en docardium

Perhaps beginning already on the 17th day and certainly manifest by the time of hatching some change must occur which decreases the susceptibility to allantoic toxin This appears from the fact (last column of Table 2) that the amount of toxin per 100 grammes embryo which kills half their num ber is about doubled on the day of hatching

Most of the newly hatched chicks that died from i v injection of AF died within 10 minutes Postmortem showed clots in the heart cavities, mostly the right atrium but no lesions in the heart muscle or the peri cordium The gross picture was on the whole very unspectacular in comparison with that seen in the embryos All the more so it was surprising to find petecchiae in the thymus where no lesions were observed in the em bryos Histology showed that these 'pe tecchiae were almost entirely due to greatly dilated capillaries in the thymic medulla

Changes in Toxicity of Allantoic Fluid during Embryonic Life

While allantoic and amniotic fluids from a 10 day embryo are almost watery clear and colourless the 17–18 day allantoic fluids are usually cloudy from precipitated urates yellowish and somewhat slimy The amniotic fluid (AMF) remains colourless but may be come slightly cloudy too and markedly more viscous

The experiment shown in Table 4 was performed in order to measure the relative potency of AF from embryos of 10 to 18 days of development and for the older embryos to compare the toxicity of AF and AMF All test preparations represented pools from at least 10 embryos and were dialysed against isotonic PBS (pH 7 3) to remove urates and then further cleared by spinning at 2000 rpm for a few minut s

in vito it was to be expected that heparin would protect the embryo

Table 5 shows that as little as 1 I U of heparin in an inoculum containing a near LD 100 dose of AF offered very marked pro tection whereas $^1/_5$ I U did not Embryos protected with heparin showed few or no petecchiae

It was found in other experiments that heparin injected *prior* to the injection of AF gave a less perfect, but still significant pro tection On the other hand even 50 I U administered from 25 seconds to 5 minutes *after* the AF failed to protect a single out of 18 embryos Admittedly control embryos injected with 50 I U of heparin alone had a 50 per cent mortality in the same 2 hour period, apparently due to bleeding from the injection site They did not show petecchial bleeding No deaths occurred from injection of 25 I U alone

Epsilon amino caproic acid (Epsikapron) which is reputed to block fibrinolysis by ac tivated plasminogen had no detectable ef fect on either mortality or the occurrence of haemorrhagic lesions

Trasylol, another anti fibrinolytic agent, gave in very high doses (200–1 000 units) a significant but far from complete protection In fact the protected embryos which were still alive 2 hours after injection showed vir tually as severe haemorrhagic lesions as those which died in the same period

Trasylol mixed with AF was also tested in the *in vitro* clotting assay in a concentra tion corresponding to the expected blood level of Trasylol after injection of 1 000 units It reduced the activity of the AF with about 30 per cent, irrespective of whether the 2 reagents were preincubated for 30 minutes at 37° or were mixed immediately before addition of paraffin plasma The sligthly pro tective effect of Trasylol in high doses might thus be due to anti coagulant properties rather than to diminished fibrinolysis

Inhibition of Toxicity with Normal Chicken Blood Preparations

As illustrated already by Tables 2 and 3, *the newborn chick is less susceptible to* al lantoic toxin than is the embryo the body weight adjusted LD 50 is higher and the haemorrhagic lesions are markedly reduced, even in fatal cases It was thought that this might reflect the natural development of a defence mechanism around the time of hatching and whatever the nature of such a defence, it was of interest to see whether it could be conferred passively by a humoral or cellular factor

Table 6 lists three independant experiments

TABLE 6 *Protection of 13 Day Embryos by Passive Transfer of Chicken Plasma or Serum*

	Pre treatment	Interval	Toxic inoculum	Deaths/injected
Exp 1	0 20 ml PBS	1 hour	0 15 ml AF	10/10
	0 20 ml adult chicken plasma	–		1/10
	0 07 ml same plasma	–		8/8
	0 20 ml serum same bird	–		2/8
	0 20 ml plasma newborn chick	–	–	4/15
Exp 2	0 30 ml citrated PBS	½ hour	0 20 ml AF	8/9
	0 30 ml adult chicken plasma	–	–	0/8
	0 15 ml same plasma			2/8
	0 30 ml 17 day embryo plasma	–		6/6
	0 15 ml same plasma	–	–	8/8
Exp 3	0 30 ml PBS	¾ hour	0 20 ml AF	9/9
	0 30 adult serum	–	–	0/10
	Same pr incubated wi h 0 20 ml AF at 37 for 50 mi utes			8/8

which all demonstrate a marked protection by 0 15–0 30 ml of citrated adult chicken plasma or serum injected i v into 13 day embryos prior to challenge with a highly toxic dose of dialysed AF from 17 day embryos

Exp 1 shows also that a pool of plasma harvested from newborn chicks (of which about half had not yet finished hatching when they were bled) was almost as effective Moreover it shows that serum protects to about the same degree as plasma thus excluding both fibrinogen and citrate Citrate alone was tested in other experiments and found of no protective value

Exp 2 shows that the plasma of 17 day embryos has no protective effect in the dose range used (0 15–0 30 ml) Thus the increase in resistance to i v injection of AF which was found to occur between the 17th day of embryonic life and the day of hatching (Table 2) is apparently correlated with the plasma concentration of the protective agent

Exp 3 illustrates the presumably important point which has been verified in several other experiments that the protective principle in chicken serum or plasma works considerably better if administered prior to the AF injection Pre incubation at 37° of the same amounts of the protective and the toxic preparations failed to show the protective effect This fact is not due to thermo lability of the protective principle in the serum as similarly pre warmed serum was fully protective when it was thereafter injected in the embryo 30 minutes before the AF In fact heating at 56° for 10 minutes had no demonstrable effect on the serum s protective power

Other experiments showed that the pre injection time which serum needed in order to give its full protective effect was of the order of minutes Thus it was found in one experiment that a 5 minutes interval was almost as good as 35 minutes whereas 15–30 seconds provided only a slight and insignificant reduction in mortality In another experiment of similar design except that adult chicken plasma was used instead of serum there was protection of half the embryos when plasma was given either together with the AF or 15 seconds before but there was protection of all but one out of 24 embryos when the plasma was injected from 5 to 80 minutes before the challenge with AF

It was also tested whether washed buffy coat cells corresponding to 0 30 ml adult chicken blood were of protective effect when injected under conditions which were optimal for protection by serum or plasma but they were not

On the Specificity of the Toxic Agent and the Inhibitor

In an early stage of this work before the toxicity of the AF had been found to be correlated with its thromboplastic effect experiments were made to see whether any individual specificity was involved In outbred embryos AF drawn from one embryo and injected both in the same egg and into another egg showed on the average no differential toxicity Likewise in partially inbred strains of chickens which were homozygous for the strong histocompatibility and blood group locus B there was no preferential toxicity of AF embryos of either the same or the opposite group

It was later found that even turkey embryos were susceptible to the toxic principle in chicken AF and died with exactly the same haemorrhagic lesion as the chicken embryos

On the other hand a very potent concentrated preparation of chicken AF, which killed almost instantaneously 3 out of 3 injected 1 day old chicks did no visible harm to newborn mice by i v injection although the latter received releated to body weight 10 times the dose which killed the chicks Therefore at least in distantly related species it seemed that AF was no longer toxic

Turning now to the specificity of the protective serum factor it appeared on the other hand that sera of a variety of mammalian species (guinea pig rabbit mouse man sheep and cow) all protected chicken em

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Trasylol mixed with AF was also tested in the *in vitro* clotting assay in a concentration corresponding to the expected blood level of Trasylol after injection of 1,000 units It reduced the activity of the AF with about 30 per cent irrespective of whether the 2 reagents were preincubated for 30 minutes at 37° or were mixed immediately before addition of paraffin plasma The sligthly protective effect of Trasylol in high doses might thus be due to anti coagulant properties rather than to diminished fibrinolysis

Inhibition of Toxicity with Normal Chicken Blood Preparations

As illustrated already by Tables 2 and 3 the newborn chick is less susceptible to allantoic toxin than is the embryo the body weight adjusted LD 50 is higher and the haemorrhagic lesions are markedly reduced even in fatal cases It was thought that this might reflect the natural development of a defence mechanism around the time of hatching and whatever the nature of such a defence it was of interest to see whether it could be conferred passively by a humoral or cellular factor

Table 6 lists three independant experiments

TABLE 6 *Protection of 13 Day Embryos by Passive Transfer of Chicken Plasma or Serum*

	Pre treatment	Interval	Toxic inoculum	Deaths/injected
Exp 1	0 20 ml PBS	1 hour	0 15 ml AF	10/10
	0 20 ml adult chicken plasma			1/10
	0 07 ml same plasma			8/8
	0 20 ml serum same bird			2/8
	0 20 ml plasma newborn chick	–	–	4/15
Exp 2	0 30 ml citrated PBS	½ hour	0 20 ml AF	8/9
	0 30 ml adult chicken plasma	–		0/8
	0 15 ml same plasma			2/8
	0 30 ml 17 day embryo plasma			6/6
	0 15 ml same plasma	–	–	8/8
Exp 3	0 30 ml PBS	¾ hour	0 20 ml AF	9/9
	0 30 adult serum	–	–	0/10
	Same preincubated with 0 20 ml AF at 37 for 50 minutes			8/8

e research tool for the study of intra ular clotting To my knowledge embryos in fact been used surprisingly little in ulation research Surprising not least be of the decisive advances in the field have repeatedly come from the in al finding of individuals with rare lary defects in the clotting system Why ume a priori that embryos are very adults and proceed to look systema or defects defined by the stages of c development?

be recalled from the data presented were three in principle different hich the lethal action of AF could ed (1) By the simultaneous ad of heparin which fact requires omment (2) by prior injection al dose of AF and (3) by in mal homologous or heterologous chanism of the protection af second procedure has not been ained However it seems a pthesis that it is due to pre blood of one or more of the which are activated by AF or the manifestation of the and toxic) effect of the

tion by normal sera which ntrigued the author First in is obvious that this re al necessity for the in tion against self compo damaged or effete cells cellular turn-over is the material one would ex and the greater the nhibitors In fact we ate a very significant content per mg wet g the course of the t versus host reaction and adult mice (Za n this co may au 66) lar

immunologist (perhaps too easily) that natural inhibitors with such a protective function may be natural autoantibodies Unfortunately quite considerable efforts to isolate and characterize the protective principle in adult chicken serum were never awarded with decisive results We thereafter turned to attempts at purifying the natural inhibitors of guinea pigs and human sera, although we knew, at described in the present paper, that these might be different agents because of the fact that they had the greatest effect on pre incubation *in vitro* with AF whereas the chicken serum inhibitor worked best when given *in vivo* prior to the toxic challenge

As it will be described in a separate publication (*Mann Jensenius Simonsen & Abildgaard* 1969) we succeeded in isolating an agent from both sources which had the dual properties of protecting chicken embryos against a lethal dose of thromboplastic lipoprotein of AF derivation and of inhibiting bovine as well as chicken thrombin *in vitro* We also found that thrombin both of bovine and chicken origin when injected i v in the chicken embryo would produce the same syndrome as caused by chicken thromboplastin Evidently thrombin has in contrast to thromboplastin little species specificity as shown also by the findings of *Chandrasekhar & Laki* (1968) The existence of an inhibitor of thromboplastin as such was not proved but has not been excluded either

The so called antithrombin III earlier isolated by *Abildgaard* in Oslo from human and bovine plasma (see *Abildgaard* 1968) proved also protective in the chicken embryo test system and seemed on further analysis to be the same factor as the guinea pig and human antithrombins isolated in Copenhagen

Antithrombins have apparently not been shown earlier to have an effect *in vivo* They belong to the general class of normal antiproteases which form an interesting group of serum proteins which are as yet little known in respect to their chemical structure They are not immunoglobulins but it re

TABLE 7 *Protective Effect of Guinea Pig Serum (G PS) Administered Before or Simultaneously with AF or Pre Incubated with AF Before Injection*

Treatment	Deaths/injected
Phosphate buffered saline 0 05 ml + AF 0 12 ml	8/8
G PS 0 050 ml injected 60 minutes before AF 0 12 ml	1/8
– 0 017 ml – – – –	7/8
G PS 0 050 ml injected simultaneously with AF 0 12 ml	1/8
– 0 017 ml – – – –	5/8
G PS 0 050 ml pre incubated with AF 0 12 ml	0/8
– 0 017 ml – – –	1/8

Pre incubation of G PS with AF was carried out for 60 minutes in a 37 water bath

bryos against a lethal dose of AF Table 7 which shows a single experiment with guinea pig serum illustrates a) that this foreign *serum is highly protective even in doses which* are about 10 times lower than the protective dose of chicken serum (cf Table 6) and b) that in contrast to chicken serum it protects better when it is pre incubated with AF *than when it is injected prior to the AF* The latter finding was verified in several other experiments

Of the various mammalian sera tested guinea pig serum seemed to be the most active protective agent

DISCUSSION

The *primary* aim of this work was to investigate the nature of the unexplained toxicity of normal allantoic fluid (AF) for the chicken embryo itself The data presented can leave *little doubt* that the toxicity is essentially due to thromboplastic material which accumulates in the allantoic sac during embryonic life *in increasing concentrations* least from the 10th to 18th day of incubation The main points in support of this conclusion are a) the close correlation found between the toxicity and the thromboplastic activity *in vitro* of different preparations of AF and b), the fact that heparin administered simultaneously with AF completely inhibited the toxicity There is additional *circumstantial evidence* to be seen in the species *specificity involved* It has been firmly established (*Astrup* 1965 *Irsigler et al* 1965) that tissue thromboplastins extracted from various organs possess a *considerable degree* of specificity Their activity is much less when tested on the plasma of distantly related species

Accepting that intravascular clotting provoked by the injected thromboplastic material is the essential feature in the described *haemorrhagic syndrome it is also clear that* the present work leaves virtually the whole of the detailed pathogenesis to be explained For one thing it is wholly unexplained why the distribution of the haemorrhagic lesions should vary as it does with the age of the embryo (cf Table 3)

The very fact that i v injection of *thromboplastic tissue* extracts carries the danger of clotting disorders is naturally an old observation It can at least be traced back to the eighties of the late century (*Wooldridge* 1887) and has been the subject of several later investigations (e g *Sneider* 1946 a & b *Thomas* 1947 *Lak & Galewsky* 1966) Furthermore, intravascular clotting caused by *excessive liberation of thromboplastic* material is in all likelihood an important factor in the dreaded defibrination syndrome encountered sometimes in pathological deliveries as well as in various cases of major surgical *interventions* (cf review by *Verstraete* & *Vermylen* 1968)

In a sense, the results presented here might therefore largely have been anticipated and the i v injection of chicken embryos might well have been devised by experimental haematologists as a cheap and easily reprodu

cible research tool for the study of intra vascular clotting To my knowledge embryos have in fact been used surprisingly little in coagulation research Surprising not least be cause of the decisive advances in the field which have repeatedly come from the in cidental finding of individuals with rare heredidary defects in the clotting system Why not assume a priori that embryos are very deficient adults and proceed to look systema tically for defects defined by the stages of embryonic development?

It will be recalled from the data presented that there were three in principle different ways by which the lethal action of AF could be countered (1) By the simultaneous ad ministration of heparin which fact requires no further comment (2), by prior injection of a sub lethal dose of AF and (3) by in jection of normal homologous or heterologous sera The mechanism of the protection af forded by the second procedure has not been critically examined However, it seems a reasonable hypothesis that it is due to pre emption in the blood of one or more of the clotting factors which are activated by AF and necessary for the manifestation of the thromboplastic (and toxic) effect of the latter

It is the protection by normal sera which has in particular intrigued the author First of all the suggestion is obvious that this re flects a physiological necessity for the in dividual for protection against self compo nents liberated from damaged or effete cells The more rapid the cellular turn over is the more thromboplastic material one would ex pect to be produced and the greater the demand on natural inhibitors In fact, we were able to demonstrate a very significant rise in thromboplastic content per mg wet weight of spleen during the course of the highly proliferative graft versus host reaction in both chicken embryos and adult mice (*Za leski* 1968) Pertinent in this context may also be the demonstration by *Saunders* (1966) of the formation of toxic products of cellular degeneration during embryogenesis

The thought comes easily to mind for an immunologist (perhaps too easily) that na tural inhibitors with such a protective function may be natural autoantibodies Un fortunately, quite considerable efforts to isolate and characterize the protective prin ciple in adult chicken serum were never awarded with decisive results We thereafter turned to attempts at purifying the natural inhibitors of guinea pigs and human sera although we knew, at described in the pre sent paper that these might be different agents because of the fact that they had the greatest effect on pre incubation *in vitro* with AF, whereas the chicken serum inhibitor worked best when given *in vivo* prior to the toxic challenge

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histological examination revealed *Toxoplasma* like organisms in heart liver lymph nodes and brain of the fetuses *Toxoplasma* as the causative agent of the outbreak was supported by the demonstration of rising titres in some gilts and by the isolation of *Toxoplasma* from a 2 month old piglet the only survivor of a litter

Becker (1952) infected an 8 month old serologically negative gilt by feeding it with mice acutely infected with a virulent strain of *Toxoplasma* The feeding was begun in the 10th week of gestation and 6 days after the first feeding the animal developed transient febrile disease Nine piglets were born a few days earlier than expected Three of them died on the 4th 9th and 12th day respectively *Toxoplasma* was isolated by mouse inoculation of pooled organs from these piglets and in two of them the parasites were seen by direct microscopy of brain and peritoneal exudate

Sanger & Cole (1955) infected three pregnant gilts with pig tissue known to be infected with *Toxoplasma* of porcine origin A combination of subcutaneous intramuscular and peroral inoculation was used and the gilts were infected on the 84th 107th and 108th day of gestation resp c tively Two of the gilts showed transient disease 4–6 days after inoculation and at term the first gilt delivered two stillborn and five live piglets while the other delivered live piglets *Toxoplasma* could not be demonstrated in nine live piglets examined from the three gilts but *Toxoplasma* was isolated by mouse inoculation from the placenta of two gilts and from the colostrum of one gilt No *Toxoplasma* was demonstrated in the gilts when slaughtered 5–7 weeks after parturition

Boch et al (1965) infected orally and/or intraperitoneally three groups of gilts and sows with *Toxoplasma* strains of porcine canine and human origin Eight animals were infected 18–278 days before pregnancy but the subsequent pregnancy had a normal course and no evidence of fetal infection could be established Seven animals were reinfected at various stages during pregnancy and four sows considered to be seronegative (DT titres 4 to 16) were infected on the 41st to the 99th day of pregnancy Pregnancy was normal in all these animals and attempts to demonstrate *Toxoplasma* in their newborn piglets gave negative results However when 10 of the sows ere slaughtered *Toxoplasma* was found in at least one organ from eight sows usually heart or br i Uterus and milk glands were negative in all 1 animals

The present paper reports the result of *Toxoplasma* infection of seronegative preg nant sows (1) with trophozoites and (2) with cysts from mouse brain Ir ord r to ensure maternal parasitaemia and to nl tr s placental passage, the trophozoites were given intravenously in large doses The cysts were given perorally also in large doses to ensure infection All sows were infected 5–6 weeks before term to allow sufficient time for abortion to occur

MATERIAL AND METHODS

Experimental animals Eight pigs were used in the preliminary experiments and six pregnant sows of the Danish Landrace breed were used for the main experiments Four pigs in each of two groups were litter mates and were 21 weeks old (Experiment 2) and 10 weeks old (Experiment 6) respectively Each group of pigs was kept in one pen The sows had a known date of conception and a history of normal pregnancies, and they were housed in separate pens During a preinoculation period of 3–13 days no fever or other clinical signs of disease were observed in the animals They were serologically negative with dye test (DT) titres of less than 5 at the time of inoculation

Inoculation Two strains of *Toxoplasma gondii* were used Trophozoites of the RH strain were used for intravenous inoculation (10–20 ml) and cysts of an avirulent porcine strain (Statens Seruminstitut Strain 119) were used for oral infection (brain suspension in saline by stomach tube) Details of strains and inoculation procedures are given in Table 1

Observations on inoculated pigs and sows The animals were observed clinically The temperature was measured twice daily for 3–5 weeks The sows were also observed for signs of impending abortion or parturition During the first post inoculation days blood was examined for *Toxoplasma* by inoculation as shown in Tables 2 and 3 Blood for serological examination was collected daily or every other day during the first 1–2 weeks and thereafter at weekly intervals At death or sacrifice all animals were necropsied and examined for gross lesions Material was examined for *Toxoplasma* by mouse inoculation and by histopathological technique as shown in Tables 2 and 3

TABLE 1 *Toxoplasma gondii Strains Employed in Experimental Porcine Toxoplasmosis with Details of the Inoculations*

Experiment number	Strain	Inoculation date	Inoculation inoculum	Inoculation route	Animal number	Size of inoculum§
1	RH*	Feb 15 1965	peritoneal exudate from mice	i v	153	2×10^{9}
2	RH	Apr 19 1966			20 19 21 22	$3\ 5 \times 10^{7}$ $3\ 5 \times 10$ $3\ 5 \times 10$ $3\ 5 \times 10^{4}$
3	RH	Mar 1 1967			2	$5\ 5 \times 10^{7}$
4	RH	Mar 30 1967			36	$6\ 9 \times 10^{6}$
5	RH	June 7 1967			44	$6\ 9 \times 10$
6	119†	Nov 22 1966	mouse brain suspension	per os	23 24 25 26	$\geqq 0\ 5 \times 10^{6}$ $\geqq 2 \times 10^{7}$
7	119	Dec 6 1967			47	$\geqq 5 \times 10^{6}$
8	119	Dec 12 1967			50	*not titrated*

* Isolated from man in 1941 USA Continuous intraperitoneal mouse passages since isolation
† Isolated from swine in 1966 Denmark Three mouse passages since isolation
§ *Based on direct count of trophozoites in Experiments 1–5* based on estimate of number of mouse infectious doses in Experiments 6–8

Observations on fetuses and piglets All liveborn piglets except Nos 9–13 from sow 47 were bled from the navel cord for isolation of *Toxoplasma* and serological examination before being allowed to suckle their dam Retained fetuses and stillborn piglets were examined for gross lesions and material from various organs was examined for *Toxoplasma* as shown in Table 4 The liver spleen lung and when possible heart blood of these animals were examined for bacteria

The liveborn piglets remained with their dam and were observed clinically until death or sacrifice 22–32 days after birth They were examined as shown in Table 4 Piglets that died during the observation period were examined in the same manner as stillborn piglets

Serological examination All blood samples were examined serologically in the dye test (DT) The test was performed as indicated by *Sabin & Feldman (1948)* and as standardized by *Aagaard* (1960) using dilutions 1 5 1 10 1 50 1 250 etc All sera were inactivated for 30 minutes at 56° C and all titres reported are final dilution titres

Parasitological and bacteriological examination Blood and tissue specimens were examined for parasites by intraperitoneal injection in *Toxoplasma* free mice bred at the institute During the acute stage of infection citrated or heparinized blood samples were drawn daily and inoculated into groups of four mice each mouse receiving 0 5–1 0 ml Cord blood from newborn pigs was examined in the same way Tissue specimens were obtained from br[illegible], heart lung and liver and in[illegible] [illegible] es also from spleen kidney me

TABLE 3 *Course of Toxoplasmosis in Pregnant S*

Sow no	Inoc on day of pregnancy	Clinical response	*Toxoplasma* isolated from blood	Serological response maximum titre	titre a farrowi or sacri
153	74	fever (2–3) anorexia (2–4) dyspnoea (2–4)	+ (2–4)		50
2	74	fever (2–6) anorexia (2–5) dyspnoea (2–9)	+ (?–7)	6?50(7)	1250(4
36	78	fever (2–7) anorexia (2–7) dyspnoea (?–12)	+ (2–10)	1250(7)	1?50(4
44	87	fever (1–6) anorexia (2–7) dyspnoea (2–8)	+ (2 4 5) 0(9–10)	6?50(9)	1?50(?
47	76	fever (4–7) anorexia (5–10)	+ (3 5–8) 0(1 ? 4 9–12)	6?50(12)	?50(1
50	76	fever (4–7) anorexia (4–9)	+ (? 4–9) 0(1 10–12)	1250(10)	?50(4

Figures in parentheses refer to day after inoculation

examined from sow 153 which died during the acute phase of infection At sacrifice of the surviving sows 25–79 days after infection no *Toxoplasma* could be demonstrated

Porcine strain infection Sows 47 and 50 showed a febrile response with maximum temperatures of 40 6 to 41 3° C after an incubation period of 4 days During the fever the animals showed lack of interest in their food and apathia but no other prominent symptoms

Toxoplasma could be demonstrated in the blood from the 2nd–3rd day to the 8th–9th [illegible] DT antibodies became demonstrable on the 6th day and maximum titres of 1250 and 6250 were reached on the 10th–12th day Evidence of impending parturition was not present in the sows until [illegible] days 28 and [illegible] on the 104th and 111th day of pregn[illegible] [illegible] and both farrowed dead and nor[illegible] piglets 2–3 days after the expected term

At sacrifice 72 and 73 days after infection the two sows still had a titre of 250 and *Toxoplasma* was d[illegible]nonstrated [illegible]n most of the organs examine

Observations on the Fetuses and Piglets

Table 4 shows the condition of the fetuses and piglets at delivery and the results of parasitological serological and bacteriological examinations Litter mates are numbered according to the order of birth and those showing identical parasitological findings are grouped together No examination was made of the fetuses from sow 153

Stillborn piglets The retained litter of 14 fetuses and 17 of the 21 stillborn piglets showed evidence of death having occurred a considerable time prior to delivery Pronounced fetal maceration (+++) characterized by dehydration with total resorption of body fluids was present in these 14 fetuses and 17 piglets

The remaining four stillborn piglets had apparently died later in pregnancy Moderate maceration (++) characterized by anasarca generalized haemolysis and a uniform greyish red colour of the abdominal organs was found in three piglets (Nos 3 9 and 15 sow 2) Slight maceration (+) characte

Outcome of pregnancy	Examination for *Toxoplasma* by inoculation/microscopy at death or sacrifice of sows				
	brain	heart	lung	liver	uterus
The sow died day 4	+/0	+/0	+/+	+/+	+/0 (4)
Parturition day 116 (42) 8 stillborn and 7 live piglets	0/0	0/0	0/0	0/0	0/0 (79)
Sow sacrificed day 118 (40) 14 retained dead fetuses	0/0	0/0	0/0	0/0	0/0 (40)
Parturition day 112 (25) 8 stillborn piglets	0/0	0/0	0/0	0/0	0/0 (25)
Parturition day 118 (42) 1 stillborn and 12 live piglets	+/0	+/0	0/0	+/0	0/0 (72)
Parturition day 117 (41) 4 stillborn and 11 live piglets	+/0	+/0	0/0	+/0	+/0 (73)

rized by serous effusions in the abdominal and thoracic cavities was seen in one piglet (No 11, sow 47)

All attempts to demonstrate viable *Toxoplasma* in the dead fetuses and stillborn piglets gave negative results but histological examination revealed intracellular proliferating forms of *Toxoplasma* in 14 of the 31 individuals with pronounced maceration in all three piglets with moderate maceration but not in the one piglet with slight maceration (see Table 4)

Normal piglets Toxoplasma was isolated from the cord blood of three of 30 apparently normal piglets One of these (No 2 sow 2) died on the 4th day of life with a titre of 50 and *Toxoplasma* was isolated from all the material examined The other two piglets (Nos 4 and 10 sow 50) remained clinically normal and were sacrificed on the 28th and 30th day of life At necropsy *Toxoplasma* could be demonstrated in all the material examined One piglet (No 7 sow 2) from which *Toxoplasma* had not been isolated from cord blood died on the 3rd day and *Toxoplasma* was isolated from all the material examined

Four other piglets (No 14 sow 2 Nos 4 7 9 sow 47) also died within the first days of life *Toxoplasma* could not be demonstrated but *Escherichia coli* and *Klebsiella* were isolated from the lungs of one piglet, another had a ruptured stomach, and in the third piglet a large amount of blood was present in the abdominal cavity No gross lesions were present in the fourth piglet

The remaining 22 piglets appeared to be clinically normal during an observation period of 23 to 32 days At necropsy *Toxoplasma* was isolated from 7 of these piglets all of which were from the two sows infected with the porcine strain

DISCUSSION AND CONCLUSIONS

The results of the present study show that *Toxoplasma gondii* strains are able to produce maternal disease and fetal infections in swine However while the infection with

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DISCUSSI CONCL

The results of the *Toxoplasma gondii* tr duce maternal d ir sw n How r w

Acta path microbiol scand Section B 78 140–148 1970

SPECIFIC UPTAKE OF HOMOLOGOUS DNA ACCOMPANYING TRANSFORMATION IN *NEISSERIA MENINGITIDIS*

SIDSEL JYSSUM and KAARE JYSSUM

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
Rikshospitalet Oslo Norway

Transformation of N meningitidis by homologous genetically marked DNA may be competitively inhibited by non marked DNA from *N sicca E coli* and calf thymus It seems that homologous DNA has higher inhibiting activity than the other DNAs tested The binding by recipient cells of radioactivity from labelled DNA preparations was measured in the transformation system An uptake of radioactivity from intact DNA which could not be removed by deoxyribonuclease treatment and repeated washings was found with homologous DNA but not with DNA from *E coli* This irreversible uptake was restricted to competent recipient cells The uptake was correlated with genetic transfer The findings indicate that the first step in DNA uptake is a reversible binding to the surface which may depend upon structures common to double stranded DNA *The following step appears to be an irreversible phase resulting in deoxyribonuclease insensitivity* This stage seems to be characterized by a specificity for homologous DNA

When little or no transformation is obtained with DNA of heterospecific origin the question of uptake must be considered It is known that bacterial cells retain DNA in at least two distinguishable states (3 9 23) Part of the DNA although substantially resistant to further washing at low temperatures is removed on merely rewarming the cells, or may be removed more rapidly by exposure to deoxyribonuclease The remainder appears to be permanently bound The specificity of the reversible binding as well as that of the irreversible phase leading to deoxyribonuclease insensitivity has been examined in several transformable species

In the case of pneumococcus any double stranded DNA of proper molecular weight seems to be bound by competent cells, and presumably taken up Calf thymus DNA competitively inhibits the uptake of pneumococcal transforming DNA (13), and ^{32}P labelled *Escherichia coli* DNA although not transforming has been found to be irreversibly bound by pneumococcus cells almost as well as the homospecific DNA (22 23)

Competent cells of *Streptococcus* do not discriminate between DNA from the closely related Challis strain from pneumococci staphylococci *E coli* and from calf thymus as expressed in transformation inhibition experiments (5 6 26)

In *Bacillus subtilis* DNA samples from a number of bacteria including those inactive in transformation compete with active DNA for receptor sites indicating that those samples which do not transform are not degraded and are capable of carrying out the initial stages of the transformation process (25) The observation that *B subtilis* cells that are competent for transformation are also competent

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for transfection by phage DNA and *vice versa* indicates that the DNA penetration process in this species is also non specific (27)

In *Haemophilus* the uptake process seems to involve an element of specificity for homologous DNA Competitive inhibition and uptake appear to be largely restricted to DNA extracted from rather closely related species DNA s from two strains of *H parainfluenzae* which do transform *H influenzae* although at very low frequency were found to inhibit whereas DNA from *B megatherium* failed to do so in spite of the fact that the latter DNA has the same density and over all composition as H *influenzae* DNA (28 29)

Previous work with *Neisseria meningitidis* transformation supports the assumption that transforming DNA enters the cell via the point in the membrane to which the replication point is attached by a mechanism involving homology (18) According to this hypothesis the uptake of DNA into competent cells should be specific for homologous DNA and for DNA which is sufficiently related to exhibit activity in transformation

The present communication deals with the specificity of DNA uptake by competent *N meningitidis* cells Attempts have been made to examine the reversible binding and the irreversible deoxyribonuclease insensitive step *separately with regard to specificity for homologous DNA*

MATERIALS AND METHODS

Strains The following mutants from the wild type strain M1 of *N meningitidis* were used as recipients during transformations and uptake experiments M1–12 *his* M1–6 *his pro* M1–18 *his gly* (15) The *str* marker (Str r) was a single step high level resistant mutant of the sensitive strain (Str s) Genetically competent variants indicated by the symbol *cp* and genetically incompetent variants indicated by *cp* were obtained and controlled as previously describ d (16) A thymine requiring mutant of *E coli* 15 and a strain of *E coli* K 12 were the same as those used in previous work (12 17)

Media Blood agar or heart infusion (HIB Difco) agar was used as solid complete medium Fluid complete medium was brain heart infusion broth (BH Difco) The basal media were those previously used (14 15) The medium used for incorporation of ^{32}P (Tris basal medium) was the sam as the basal medium (14) except that the K phosphates were omitted and it was made 20 mM with Tris HCl buffer pH 7 4 The minimal media for *E coli* were those previously used (12)

Genetic procedures Transformations were performed according to the previously described technique (15 17) DNA was either prepared as previously described (4 15) or by a phenol extraction method (21)

Inhibition of transformation The inhibition of tran formation by unmarked DNA was tested in a transformation system containing 1 4 ml HIB with 0 0015 M $CaCl_2$ (15) 0 2 ml of receptor cell suspension in saline 0 3 ml of a mixture of transforming DNA and inhibiting DNA in NaCl citrate buffer The receptor culture was inoculated from an over night culture on blood agar and grown in BH until the logarithmic phase (Absorbance 0 2–0 4) The cells were harvested in the centrifuge resuspended in saline to approximately 10^9 colony forming units per ml and inoculated into the transformation system After incubation at 37 C with shaking for 20 minutes the DNA was add d Transformation was terminated after the desired time usually 30 minutes by the addition of 0 1 ml deoxyribonuclease (DNase) giving a final concentration of 50 µg/ml (15)

The relative inhibiting concentration of DNA was calculated according to the principles sketched out by *Schaeffer et al* (29) The concentration of inhibiting DNA was called n and was expressed with the concentration of transforming DNA as the unit For each value of n corresponds a recidual transforming activity R = tr /tr × 100 Since the inhibition is competitive the concentration n of receptor (homologous) DNA which under the same conditions would give the same recidual transformation is n = (100 R)/R The inhibiting activity of the DNA considered relative to the receptor (homologous) DNA is then expres ed by the value n/n

Preparation of ^{32}P labelled DNA A start culture in complete medium was inoculated from an over night culture on blood agar and grown to the *logarithmic growth phase Tris* basal medium (6 ml) was inoculated with 0 5 ml of the culture and incubated with shaking until the absorbance had reached approximately 0 25 The culture was then mixed with prewarmed medium consisting of 30 ml Tris basal salts 30 ml HIB and 2 ml ^{32}P ortophosphate containing 4 mC The culture was incubated at 3 C with shaking At the end of the logarithmic growth phase the cells were centrifuged wa hed twice and utilized for DNA extraction

DNA was either prepared by the procedure of *Marmur* (24) or by a modification of this procedure (4) In some preparations treatment with pronase wa also included in the procedure Initial specific radioactivity of ^{32}P DNA *preparations ranged* from 2×10^4 to 6×10^4 cpm/µg DNA

TABLE 2 *Labelled Material Used to Measure the Uptake of DNA in N meningitidis*

Source of material	Batch No	Isolation procedure	RNA µg/ml	DNA µg/ml	RNA/DNA
M1 *cp* Str r	1	A	2390	190	12/1
M1 *cp* Str r	2	A	2141	359	6/1
M1 *cp* Str r	3	B without precip with isopropanol	825	325	2.5/1
M1 *cp* Str r	4	B with 2 precip with isopropanol	272	327	0.8/1
M1 *cp* Str r	5	B with 6 precip with isopropanol	46	154	0.3/1
E coli 15	1	B with 1 precip with isopropanol	0	355	

A = Modification of the procedure of Marmur (24) as used in transformation experiments (4 15)
B = Procedure according to Marmur (24)

TABLE 3 *Sorption of Tracer from Labelled DNA Preparations by Competent and Incompetent Variants of N meningitidis*

Strain used as receptor	DNA used	Labelled with	Sorption of tracer in terms of µg DNA/mg N			
			Complete system	*Blank 1* DNA + DNase at end of expt	*Blank 2* DNA + DNase at start of expt	Irreversible uptake from intact DNA
6 *his pro cp*⁺	M1 *cp* Str r (Batch 2)	^{32}P*	1.43	0.82	0.96	0.47
6 *his pro cp*	M1 *cp* Str r (Batch 2)	^{32}P	0.60	0.59	0.49	Insignificant
6 *his pro cp*	*E coli* 15 (Batch 1)	^{3}H†	0.09	0.03	0.08	Insignificant
6 *his pro cp*⁻	*E coli* 15 (Batch 1)	^{3}H	0.05	0.05	0.02	Insignificant

* DNA not specifically labelled
† DNA specifically labelled in thymine

TABLE 4 *Irreversible Uptake of Homologous DNA by Competent Cells of N meningitidis*

Expt No	DNA conc µg/ml	Transf per col f unit	µg DNA per col f unit	µg DNA per transf
1	4.1	1.5×10^{-3}	9.5×10^{-11}	6.5×10^{-8}
2	2.5	8.3×10^{-4}	4.6×10^{-11}	5.6×10^{-8}
3	2.5	3.1×10^{-4}	1.3×10^{-11}	4.6×10^{-8}

that *it cannot* be removed by DNase treatment and repeated washing The irreversible uptake seems to occur only with homologous DNA and it is restricted to the genetically competent variant It also coincides with the appearance of transformants No irreversible uptake may be demonstrated with *E coli* DNA which has very nearly the same overall base composition as the *N meningitidis* DNA

Correlation between DNA uptake and transformation The irreversible uptake of radioactive material from DNA by the competent mutant 6 *his pro cp* was related to the number of transformants in several experiments (Table 4) The quantity taken up per transformed cell was nearly the same in all the experiments

The correlation between DNA binding and genetic transfer was further examined in experiments with mutants representing three different variants with regard to genetic competence (16) No transformation and no irreversible uptake could be found in the incompetent variant 12 *his cp* In the compe

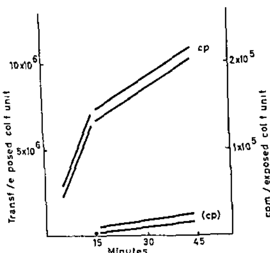


Fig 3 Transformation (solid circles) and irreversible uptake of DNA (open circles) as a function of the duration of exposure of the cells to DNA Competent recipients indicated by *cp* and recipients with defective competence by (*cp*)

tent variant 12 *his cp*⁺ the irreversible uptake of homologous DNA was almost precisely correlated with genetic transfer (Fig 3) The experiments with the variant 18 *his gly* (*cp*) which is characterized by a defective competence (16) also showed a correlation between the irreversible uptake of ³²P from intact DNA and the transformation efficiency Thus the reduction of transformation efficiency which is observed in this recipient strain when compared with the *cp* variant may probably be due to a decreased uptake of transforming DNA

DISCUSSION

The relationship between DNA concentration and the extent of transformation found in *N meningitidis* (Fig 1) has been obtained in most transformable species (1 2 5) This type of titration curve has been interpreted to mean that one molecule of DNA is sufficient for transformation to take place and that at concentrations greater than a certain limit usually between 0 1 and 1 μg DNA per ml the average recipient is receiving more than one molecule of DNA bearing the marker in question An exception is the Challis strain of *Streptococcus* (5) in which an enhancement of transformation occurs when the cells receive more than one molecule of DNA This particular pattern coincides with a DNA helping effect during transformation which may be due to the existence in the cell of a cell bound inactivator of unintegrated fragments of donor DNA (6)

Since no helping effect of DNA seems to exist in the *N meningitidis* transformation system, the affinity of various DNA s for the hypothetical receptor sites on the bacterial surface could be measured by experiments based on competitive inhibition (29) The effect of various concentrations of homologous unmarked DNA on the transformation is also that expected from a competitive inhibition by a molecular species with the same activity as the transforming DNA

All the DNA samples tested obviously compete with homologous DNA under saturating conditions indicating that those from calf thymus and *E coli* which do not transform are capable of carrying out the initial stages of the transformation process But it seems that the inhibiting activity of homologous DNA is higher than those of the DNA s from *E coli* calf thymus and *N sicca*

It should be stressed however, that the calculation of the inhibiting activities relative to the transforming DNA has been based on the assumption that the DNA s tested are of exactly the same molecular weight The variation in molecular weights must presumably be greater than that actually observed between bacterial species in order to explain the differences observed in the experiments (29) but the isolation procedures may have deleterious effects on the DNA with changes in average molecular weight and biological activity It is of interest that *Barnhart & Herriott* (3) found that the reversible binding of DNA is considerably more effected by sonication than the irreversible uptake This could perhaps explain why homologous DNA is always more effective as inhibitor than heterologous preparations regardless of the extraction procedure and of ageing

But even if the differences observed be

TABLE 1 *Influence of the Batch of* M pneumoniae *Antigen and Its Storage on the Results of the FAT*

Batch of antigen No	Age in months (average)	Number of observations	Titre of standard serum
83	9–22 (21)	2 11	1280 2560
89	1–3 (2)	7 1 1	2560 5000 10000
90	1–7 (3)	1 7 1	1280 2560 5000
91	2–4 (3)	9	2500
		40	2600*

* Geometric mean titre

2) The rack (that holds 45 slides) is immersed in a bath with distilled water at 78° C The water is heated immediately to 80° C When the agar turns opaque and starts to slip off, the rack is shaken until all the agar blocks have loosened The rack is then transferred to another water bath at 90° C for a quick washing of the slides These are placed on blotting paper so that the colonies dry by air After submergence in acetone *pro analyse* for 10 minutes the preparations are checked by microscopy for fixation of the



Fig 1 Transf ocks to mic

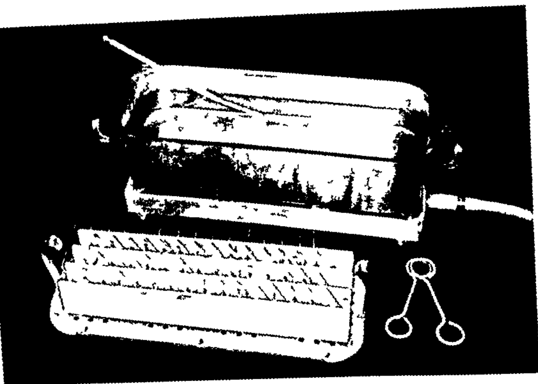


Fig 2 Water bath for rack with slides

colonies Generally more than 80 per cent are usable About 400 specimens can be handled per day The preparations can be stored in containers at 4° C for more than a year without losing their specific reactivity in the FAT B fore use the colonies are re hydrated in phosphate buffered saline pH 8

The succeeding steps of the FAT have been described previously (3 4) It can be added that the same batch of fluorescein iso thiocyanate conjugated anti human globulin from horse has been used since 1962 in al most all studies with the FAT (Lot 3112, Progressive Laboratories Baltimore) Since 1966 abso ption of the conjugate has been omitted and instead the conjugate is used at a higher dilution which gives results si milar to those obtained previously Further more 10 per cent heat inactivated (56°C/30 min) horse serum in phosphate buffered aline pH 8 was introduced as diluent for all sera to be tested This was shown to in hibit occasional non specific staining of co lonies and remnants of agar Such staining is possibly due to 1) reacti n between horse serum of the agar medium trapped during preparation of the antigen and the human sera to be tested 2) direct non specific binding of the conjugated horse serum to the antigen or 3) non specific binding between more heavily labelled molecules of the conju gate and proteins of the antigen preparation Whatever the reason may be horse serum in the diluent tends to eliminate the e unwanted reactions

The influence of different antigen pre- parations and their storage on the results of the FAT was evaluated as follows – During the past two years four different batches of antigen have been used after storage of be tween one and 22 months With these pre parations we have 40 FAT observations on titrations of the serum included as positive standard in routine tests (Table 1) There was no signi icant difference between titres ob ained with the four antigens The standard deviation of the titre values for all observa tions was 0 5 (log value) This means that, under the conditions described the titre deviates from the true value by a factor more

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		1	5000
		1	10000
90	1-7 (3)	1	1280
		7	2560
		1	5000
91	2-4 (3)	9	2500
		40	2600*

* Geometric mean titre

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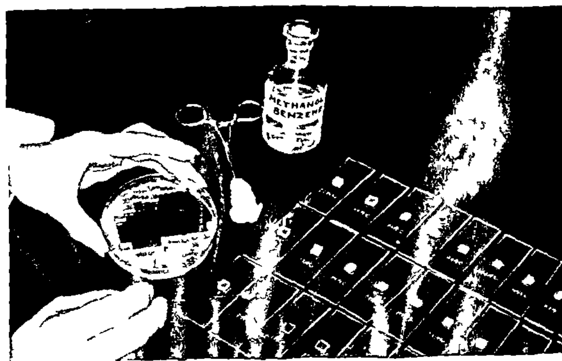


Fig 1 Transferring of agar blocks to microscope slides

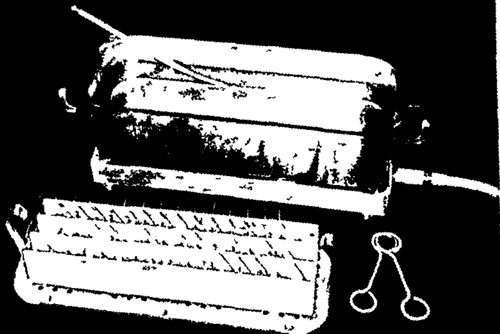


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labelling with FITC at three different dye to protein ratios (i e amount of FITC added in relation to amount of protein present) The greatest recovery of chromatographically unaltered γG globulin was obtained after labelling with 8 mg FITC/g protein (first peak, Fig 1) A ratio of 20 mg FITC/g protein gave a similar result whereas a ratio of 50 mg FITC/g protein yielded significantly less unaltered γG globulin Correspondingly much more protein was eluted by the salt gradient in the latter case (see second peak Fig 1)

The eluates from each conjugate were pooled to give four fractions Fraction I corresponds to the first peak in Fig 1 and it appeared in the first few tubes as a brilliant yellow solution which was eluted by the initial 0 01 M phosphate buffer pH 7 3 The broader peak, which appeared after application of the salt gradient was divided into three fractions Fraction II represented the ascending limb fraction III the plateau or middle part of the curve and fraction IV the descending limb Fraction I could be used directly for staining in FAT, in all other cases the fractions were concentrated to a volume of 1–2 ml before examination in FAT by immunoelectrophoretic analysis, and for specific antibodies to *N gonorrhoeae* measured in the gonococcal complement fixation test

Specific and Non specific Staining in FAT

Table 1 shows the degree of specific staining of *N gonorrhoeae* obtained with the different conjugates No brilliant staining was achieved with any of the fractions obtained from the conjugate labelled at the ratio of 8 mg FITC/g protein This also applied to the conjugate itself Brilliant staining was obtained with the conjugate labelled at the ratio of 20 mg FITC/g protein before chromatography and with fraction I obtained from this conjugate after chromatography When the most heavily labelled conjugate (*50 mg FITC/g protein*) was examined the gonococci were stained brilliantly by the conjugate and by all four fractions

Table 2 illustrates the non specific staining of leucocytes exhibited by the same conjugates and their fractions after chromatography In

TABLE 1 *Specific Staining of* N gonorrhoeae *by FITC Labelled Rabbit Anti Gonococcal Globulin before and after Chromatography on DEAE Cellulose*

Dye to protein ratio during conjugation	Before chromatography	Fractions after chromatography I	II	III	IV
8 mg FITC/g protein	+ +	+ +	(+)	(+)	(+)
20 mg FITC/g protein	+ + + +	+ + + +	+ +	+ +	+
50 mg FITC/g protein	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Symbols for degree of fluorescence see text

TABLE 2 *Non-Specific Staining of Human Leucocytes by FITC Labelled Rabbit Anti Gonococcal Globulin before and after Chromatography on DEAE Cellulose*

Dye to protein ratio during conjugation	Before chromatography	Fractions after chromatography I	II	III	IV
8 mg FITC/g protein	+ + + +	(+)	+	+ +	+ + +
20 mg FITC/g protein	+ + + +	+	+ +	+ + +	+ + + +
50 mg FITC/g protein	+ + + +	+	+ +	+ + + +	+ + + +

Symbols for degree of fluorescence see text

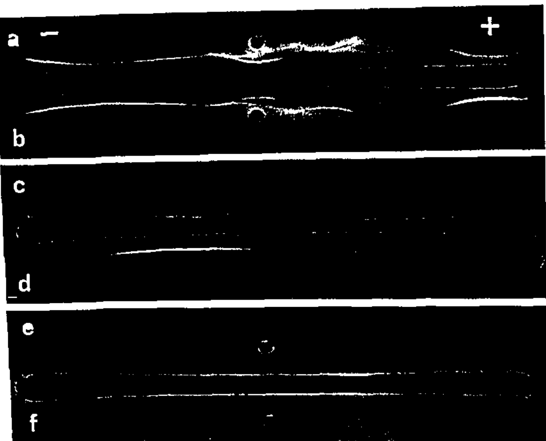


Fig 2 Immunoelectrophoretic analysis of crude rabbit anti gonococcal globulin before (a) and after (b) labelling with 50 mg FITC/g protein The protein components of fractions obtained by chromatography on DEAE cellulose are shown on the two other slides fraction I = d fraction II = c fraction III = e fraction IV = f The longitudinal trough on all the slides contained goat antiserum against rabbit serum.

contrast to the specific staining which was always correlated with fraction I non specific staining was predominant in fractions III and IV fraction I did not stain the cytoplasm of leucocytes

Fraction I from the conjugate labelled with 20 mg FITC/g protein was therefore used in experiments with smears of urethral discharge from male patients Gram stained smears from these patients had shown typical gram negative intracellularly situated diplococci Although the cytoplasm of leucocytes now remained unstained intracellular gonococci were still not visible and the extracellular gonococci were not always stained as brilliantly as those in control smears made from a pure culture of *N gonorrhoeae*

Reactive strains of *S aureus* were stained with exactly the same fractions of anti gonococcal conjugate as the gonococci (see Table 1) The *non reactive* staphylococcal strains did not stain with any of the conjugates or fractions obtained by chromatography

Immunoelectrophoretic Analyses and Gonococcal Complement Fixation Test

As shown in Fig 2 (a) the crude globulin preparation contained a variety of serum proteins after labelling with FITC an almost identical pattern was found (Fig 2 (b)) After chromatography of the conjugate on DEAE cellulose it was found that fraction

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Acta path microbiol scand Section B 78 163 175 1970

SEROTYPING OF *PSEUDOMONAS AERUGINOSA*

2 *Results of an O Group Classification*

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An account is given of the distribution of 958 strains of *Ps aeruginosa* among 14 O groups investigated by means of a tube agglutination technique and heat treated antigen suspensions Use has been made of unabsorbed O sera corresponding to *Habs* 12 O groups an O group 13 from *Sandvik s* material and an O group 14 prepared with a strain from the present material 75 per cent of the strains which originated chiefly from patients in a hospital environment could be O group determined 55 per cent of the strains belonged to O groups 6 2 5 3 and 11 Strains belonging to O groups 2 5 and 6 were the ones most frequently occurring in a hospital environment. The results showed that strains belonging to O groups 2 and 5 were more frequent than O group 6 strains in a hospital environment and that they colonized more easily than O group 6 strains wounds urinary tract ears pleura and lower respiratory tract whereas strains belonging to O groups 2 and 5 and O group 6 were equally frequent in the upper respiratory tract and in the large intestine (stools) The frequent occurrence of strains belonging to O groups 2 and 5 in samples from blood and central nervous system can be explained by the finding of these O group strains at sites from where *Ps aeruginosa* is presumed to advance into the circulation and central nervous system

Since the publication of the work of *Habs* in 1957 (5) more papers have been published both from Western and Eastern Europe from USA and from China dealing with O type classification of greater materials on *Pseudomonas aeruginosa*

Investigations have been carried out with strains isolated from animals (15 18 23) and with strains mainly isolated from human subjects (1 3 5 9 10, 11 13 14, 20, 21 22) as well as with strains isolated from surface waters (3) The different typing schemes employed from five (1) up to 25 O groups of the bacteria (23) In the present investigation 958 strains of miscellaneous origin have been subdivided into O groups using a tube agglutination technique Use has been made of 13 O group sera corresponding to strains isolated by *Habs* (5) and *Sandvik* (16) and of a further number of sera produced with strains from this material The production of O sera has been described in a previous publication by the author (12)

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11

MATERIAL AND METHODS

Strains

The examined material com[illegible] 1054 strains 338 stra[illegible] have been bioch[illegible]ally investigated

TABLE 4 *Homologous and Heterologous O Agglutinin Titres Obtained with S*

Serum	PJ 101	PJ 102	PJ 103	PJ 104	PJ 105	PJ 106	PJ 107	PJ 108	PJ 109
	1	2A	3	4	5A	6	7	8	9
O se Sandvik II*	0§	0	0	0	0	0	0	0	0
O se PJ 433	50	50	50	50	50	50	50	25	100
O se PJ 340	0	200	0	0	100	0	0	0	0
O se PJ 284	25	25	0	0	100	0	0	0	0
O se PJ 448	25	50	0	0	800	25	0	0	0
O se PJ 400	0	0	0	0	0	0	0	0	0
O se PJ 434	0	0	0	0	0	0	0	0	0
O se PJ 443	0	0	0	0	0	0	0	0	0

* Mixed O sera from animals immunized with strain Sandvik II

TABLE 5 *Distribution of 958* Ps aeruginosa *Strains Accord*

Origin and source of cultures	Total no	1	2A	2B	3	4	5A	5B 5C
1 Collections[a]	62	8	2	0	8	3	1	1
2 Surface water[a]	21	3	1	0	5	0	2	0
3 Animals[b]	16	1	0	0	0	5	0	0
4 Hospital strains not isolated from ptt	15	1	1	1	0	0	3	0
5 Hospital strains from pt material	761							
Blood	220	8	39	7	14	2	12	7
Spinal fluid (CNS)	13	0	0	1	0	0	3	0
Pleura exudate	31	0	2	3	0	0	5	2
Upper resp tract[c]	36	5	2	0	[illegible]	0	2	1
Lower resp tract[d]	64	3	8	3	[illegible]	0	3	2
Urine	118	2	21	1	6	0	7	5
Stools[e]	53	7	3	3	7	0	4	2
Wounds (kind not stated)	109	12	15	5	10	0	13	3
Ears	43	1	0	5	1	1	6	0
Organs operations	26	1	1	2	1	0	2	0
Organs autopsies	50	3	3	2	5	0	5	0
Strains non hosp ptt[f]	57	3	3	3	3	1	6	2
Patient strains with inadequate information	24	1	3	0	0	0	1	0
Total number	958	59	104	36	71	12	75	25
Frequency of O groups (%)	100	6 2	14 6		7 4	1 3	10 4	

[a] See *Jessen* (5) [b] Dogs and cats isolates from wounds and ears
[c] *Secretion from nose throat and mouth* [d] Sputum and tracheal secretion
7 samples from children < 1 year of age O groups 1 & 3 1 strain each O group 6
strains negative in O 1-14 2 strains

(·epared with 7 Strains from the PJ Collection and the O Group II Strain of Sandeił

	Strain									
	PJ 118	PJ 119	Sandv 11	PJ 433	PJ 340	PJ 284	PJ 448	PJ 400	PJ 434	PJ 443
	O groups									
	11	12	13	14	2B	5B	5C	10B	10C	10D
	0	0	1600	0	0	0	0	0	0	0
	50	25	50	6400	50	25	25	50	50	50
	0	0	0	0	800	100	100	0	0	0
	0	0	0	0	?5	800	1600	0	0	0
	25	0	0	0	?00	800	3?00	0	0	0
	0	0	0	0	0	0	0	400	100	100
	0	0	0	0	0	0	0	200	800	200
	0	0	0	50	0	0	0	?00	200	1600

§ Agglutinin titres obtained with antigen C

...to O Groups and Origin Figures Indicate Number of Strains

	O group*											No reaction in O 1–14	Antigen unstable
	2/5	6	7	8	9	10A	10B 10C 10D	11	12	13	14		
	?	16	0	1	1	0	0	6	0	0	0	2	11
	0	4	0	1	0	0	2	1	0	1	0	3	0
	0	2	0	2	0	0	0	0	0	1	0	0	5
	0	1	0	0	1	0	0	1	0	0	0	4	?
	3	23	0	8	10	3	?	1?	0	0	0	37	33
	0	7	0	0	0	0	0	0	0	0	1	1	0
	1	6	0	1	0	0	1	3	0	0	0	4	3
	0	5	0	1	2	0	1	4	0	0	0	7	0
	0	18	0	3	1	2	3	4	0	1	0	5	3
	1	13	0	0	1	2	2	9	0	0	4	12	32
	0	12	0	0	1	1	1	4	0	0	0	7	1
	?	11	0	2	1	2	4	9	0	0	1	15	6
	0	7	0	3	0	0	0	3	0	0	3	5	8
	0	7	0	1	2	3	0	1	0	0	0	3	2
	0	9	0	4	1	3	3	1	0	0	0	6	3
	0	12	0	1	4	1	4	3	0	0	0	8	3
	1	5	0	0	0	3	0	0	0	0	0	4	6
	8	156	0	28	25	20	23	61	0	3	9	125	118
	0 9	16 3	0	? 9	2 5	4 5		6 4	0	0 3	0 9	13 1	12 3

† Strains from throat swabs and whooping cough cases (21) urine (10) stools (9) and medicolegal autopsies (17) Stools Two samples from children < 1 year of age O group 4 1 strain negative in O 1 14 1 strain

* See text under RESULTS Preparation of new O group sera

TABLE 6 *Frequency of O Groups of* Ps aeruginosa *Strains from Hosp*

Hospital	Total no	1	2A 2B	3	4	5A 5B 5C	2/5
A	56	3 7§	19 6	7 1	0	10 7	0
B	34	5 9	14 7	14 8	0	5 9	0
C	47	6 4	10 6	4 3	0	14 9	0
D	72	4 4	34 7	6 9	1 4	9 5	0
E	27	3 7	11 1	3 7	0	37 1	0
Total number from 16 hospitals	503	34	90	29	1	56	3
Frequency of O groups (%)	100	6 7	17 9	5 8	0 2	11 1	0 5

* See text under RESULTS Preparation of new O group sera

inated from persons from whom more strains belonging to the same O group had been iso lated On the basis of origin the strains were divided into seven sections 763 strains isolated from patients in a hospital environment have been further divided according to sample categories Where more strains were isolated from a patient but from different sorts of material all have been included so that 763 strains in section 5 have been isolated from 731 patients The 16 strains in section 3 were from 15 animals When dividing the strains according to sample category it was not taken into consideration whether the strains had been isolated from mixed or pure culture, as the information did not permit such evalua tion With a few exceptions samples from blood and CNS contained *Ps aeruginosa* as pure culture

Strains from hospitalized patients could be distributed in such a way that 66 per cent originated from 16 central hospitals whereas

TABLE 7 Ps Aeruginosa *Strains from Hospitalized and Non Ho pitali ed Patien s*

	Material or site of isolation	Total no	1	2A 2B	3	4	5A 5B 5C	2/5
5*	*Hospital strains*							
	Total number	763	42	126	55	3	81	5
	Blood and CNS	233	3 4†	20 2	6 0	0 9	9 1	1 3
	Pleura exudate & lower resp tract	95	3 1	16 8	5 3	0	12 6	1 1
	Upper resp tract	36	14 0	5 5	16 1	0	8 3	0
	Urine	118	1 7	18 6	5 1	0	10 2	0 9
	Stools	53	13 2	11 3	13 2	0	11 3	0
	Wounds	109	11 0	18 3	9 1	0	14 [illegible]	0
	Ears	43	2 3	11 6	2 3	2 3	13 9	0
	Organs operations and sectio	76						
	Freque y of O groups (%)	100	5 5	16 5	7 2	0 4	11 0	0 7
6	Throat abs stools *non hospitali ed pts*	[illegible]0	10 0†	6 6	10 0	3 3	13 4	0

Classification number (see Table 5)

e text under RESULTS repara on of new O group sera

amples from Patients in 5 of 16 Central Hospitals

	O group*									
	6	7	8	9	10A 10B 10C 10D	11	12	13	14	
	10 8	0	7 1	1 8	7 1	8 9	0	0	1 8	
	23 5	0	2 9	0	0	2 9	0	0	[illegible]9	[illegible]
	17 0	0	6 4	4 3	0	8 5	0	0	0	[illegible]
	5 5	0	1 4	1 4	8 3	9 7	0	0	1 4	[illegible]
	11 1	0	0	0	0	0	0	0	3 7	[illegible]
	76	0	15	14	23	35	0	1	6	[illegible]
	15 1	0	3 0	2 8	4 6	7 0	0	0 2	1 2	[illegible]

§ Figures denote frequency of O group strains in per cent

the rest were evenly distributed among 72 smaller hospitals As local accumulations of strains belonging to particular O groups might be found in the 16 hospitals and this might influence the O group distribution (*Table 5*) the distribution of strains within each of the 16 hospitals was investigated Table 6 shows the O group distribution from five central hospitals designated A B C D and E There is no accumulation of particular O group strains in hospital A Accumulations of O group 2 strains were found in t[illegible] of O group 3 strains in one h[illegible] group 5 strains in one hosp[illegible] group 6 strains in three hosp[illegible] there was an accumulation of [illegible] O group strains in one hospital

Table 7 shows the distributi[illegible] of O [illegible] of strains of *Ps aeruginosa* express[illegible] centages in different categories of [illegible] samples Samples from non hospital[illegible] tients have also been included Stra[illegible]

Frequ ncy of O Groups According to Nature of Material

	O group§										
	6	7	8	9	10A 10B 10C 10D	11	12	13	14	No reaction in O 1–14	Antigen unstab[illegible]
	118	0	23	19	33	50	0	1	9	104	91
	1[illegible] 9	0	3 4	4 3	2 2	5 1	0	0	0 4	16 3	14
	25 2	0	4 2	1 1	6 3	7 4	0	1 1	0	9 5	6 3
	14 0	0	2 8	5 5	2 8	11 1	0	0	0	19 4	0
	11 0	0	0	0 9	3 4	7 7	0	0	3 4	10 1	27 0
	[illegible]2 6	0	0	1 9	3 8	7 6	0	0	0	13 2	1 9
	10 9	0	1 8	0 8	5 4	8[illegible]	0	0	0 8	13 7	[illegible] 4
	16 3	0	7 0	0	0	7 0	0	0	7 0	11 6	18 7
	15 5	0	3 0	2 5	4 3	6 6	0	0 1	1 2	13 6	11 9
	20 0	0	3 3	6 7	6 6	0	0	0	0	[illegible]0 1	0

† Figures denote frequency of O group strains in per cent

Hospital	Total no	1	2A 2B	3	4	5A 5B 5C	?/5
A	56	3 7§	19 6	7 1	0	10 7	0
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C	47	6 4	10 6	4 3	0	14 9	0
D	72	4 4	34 7	6 9	1 4	9 5	0
E	27	3 7	11 1	3 7	0	37 1	0
Total number from 16 hospitals	503	34	90	29	1	56	3
Frequency of O groups (%)	100	6 7	17 9	5 8	0 ?	11 1	0 5

* See text under RESULTS Preparation of new O group sera

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5*	*Hospital strains*							
	Total number	763	4?	126	55	3	8?	5
	Blood and CNS	?33	3 4†	20 ?	6 0	0 9	9 4	1 3
	Pleura exudate & lower resp tract	95	3 1	16 8	5 3	0	12 6	1 1
	Upper resp tract	36	14 0	5 5	16 6	0	8 3	0
	Urine	118	1 7	18 6	5 1	0	10 ?	0 9
	Stools	53	13 2	11 3	13 2	0	11 3	0
	Wounds	109	11 0	18 3	9 1	0	14 6	0
	Ears	43	2 3	11 6	? 3	2 3	13 9	0
	Organs operations and sectio	76						
	Freque y of O groups (%)	100	5 5	16 5	7 2	0 4	11 0	0 [illegible]
6*	Throat swabs stools non ho pitali ed pts	30	10 0†	6 6	10 0	3 3	13 4	0

Classification number (see Table 5)

e text under RESULTS Prepara on of new O group sera

amples fr

O group*		
6	7	
10 8	0	
23 5	0	25
17 0	0	6 [illegible]
5 5	0	14
11 1	0	0
76	0	15
15 1	0	3 0

§ Figures denote frequency

the rest were evenly
smaller hospitals As
strains belonging to particu
be found in the 16 hospitals
fluence the O group distri
the distribution of strains
16 hospitals was investigated
the O group distribution
hospitals designated A B C
is no accumulation of partic
strains in hospital A Accor

Frequency of O Groups According to

O group§					
6	7	8	9	10A 10B 10C 10D	
118	0	23	19	33	
12 9	0	3 4	4 3	2 2	
25 2	0	4 2	1 1	6 3	
14 0	0	2 8	5 5	2 8	
11 0	0	0	0 9	3 4	
22 6	0	0	1 9	3 8	
10 9	0	1 8	0 8	5 4	
16 3	0	7 0	0	0	
15 5	0	3 0	2 5	4 3	6 6
20 0	0	3 3	6 7	6 6	0

† Figures denote frequency of O group strain in per cent

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lated from organs during operations or autopsies have not been included in the calculation as these categories were too heterogeneous Furthermore, it was not known whether an *infection with Ps aeruginosa might* have been the provoking cause of death in cases where strains were isolated during an autopsy In *the* case of *non hospitalized* patients only strains from persons not suspected of suffering from infection with *Ps aeruginosa* were included

It can be seen that the O group distribution for the whole material is as follows according to a declining degree of frequency 6 2 5 3 11 1 10 8 9 4 14 2/5 and 13 O groups 7 and 12 were not represented

Among the strains from collections one is *the proposed neotype strain for Ps aeruginosa* ATCC 10145 here designated PJ 47 The strain belonged to O group 6 (17) Most of the strains from surface waters belong to O group 3 a few to O group 1 the others being dispersed among seven different O *groups* 40 *per cent of strains* isolated in a hospital environment but not from patients belong to O groups 2 5 and 6 the others being *dispersed among three different* 0 groups 25 per cent of strains from persons outside a hospital environment belong to O groups 2 and 5 21 per cent belong to O group 6 the rest being dispersed among eight different O groups 7 out of 9 strains from faeces belonged to O groups 1 2 3 4 5 and 6 whereas two strains could not be grouped

Strains from patients in a hospital environ*ment (section 5* Tables 5 and 7) show the following O group distribution 85 per cent of strains from CNS belong to O groups 2 5 nd 6 38–61 *per* cent of strains from blood pleura lower respiratory tract urine stools wounds ears and organs also belong to O roups 2 5 2/5 and 6 The distribution of strains among the other O groups is quite uniform from one category of samples t the other and in such a way that most strai are dispersed among O groups 11 3 and 1 However samples from the upper respiratory tract stools and wounds contain comparatively many strains belonging to O groups 1 and 3 31 per cent of strains from the upper respiratory tract belong to O groups 1 and 3 whereas only 28 per cent *belong to* O *groups* 2 5 and 6 It will be seen (Table 7) that samples from blood and CNS urine wounds and ears and to a lesser degree *from pleura* and the lower respiratory tract contain many more strains belonging to O groups 2 5 and 2/5 than to O group 6 whereas *samples from* the upper respiratory tract and faeces contain the same number of strains belonging to O groups 2 and 5 as to O group 6

Strains isolated from urine give comparatively often unstable antigens

As stated 60 *strains (section* 5 Table 5) were investigated serologically immediately after isolation in order to examine whether the possibility of *preparing* a stable antigen is the same whether freshly isolated or stored strains are used These strains were tested in 20 sera 63 per cent of the strains belonged to known O groups They agglutinated to the same titre as strains subcultured from ampoules or agar stabs 15 per cent of the strains gave unstable antigens whereas the rest could not be placed in known O groups

DISCUSSION

*The vaccination procedure proposed in a pr*vious paper (12) has proved to be useful as the sera prepared for this investigation were specific and *showed a fairly high homologous* titre The specificity of the O sera employed was also proved by the fact that it was pos*sible to identify* 75 per cent of the tested strains without using absorption experiments Out of 309 PJ strains tested in O group sera 1–14 by means of antigen D there were some which could not be O group identified but 9 of these could be identified *when antigen C* was used This means that antigen D was unfit as an agglutinating suspension for about 3 per cent of the strains *The insufficient ag*glutinability of these strains may be caused by O agglutination inhibition which is neutralized by boiling *for a longer time* The fact that 22 per cent of 60 freshly isolated strains could not be grouped in contrast to only 13

per cent of strains which had been stored for a long period might also be explained by O agglutination inhibition but this has not yet been investigated 12 per cent of the strains gave unstable antigens, however some of them could be identified but the results will not be discussed here *Habs* found that 10 per cent of the strains gave unstable antigens (5) The frequency of strains giving unstable antigens was the same in a material of 60 freshly isolated strains as in the material of stored strains so it will not be necessary to prepare an antigen just after a strain has been isolated

Cross reactions between O groups 1 and 6 O groups 2 and 5 O groups 4 and 11 and O groups 7 and 8 have been demonstrated The distribution of strains into O groups 2A and 2B Ogroups 5A 5B and 5C and O group 2/5 justifies the subdivision of O groups 2 and 5 Strains from the SM collection which after testing in O group sera 1–12 could be placed in groups 2A or 5A have also been tested in O group sera 2B 5B and 5C 19 of the strains belonging to group 5A gave heterologous reactions with group 2B One third of the cross reacting strains agglutinated to a higher titre in O group serum 2B than in O group serum 5A A more exact grouping of these strains included in this work under O group 5 cannot be performed until the cross reactions between O groups 2 and 5 have been elucidated by means of absorption experiments Absorption experiments will also be necessary to ascertain whether the freshly prepared sera belonging to O groups 2, 5 and 10 may be used for analysis of O factors or whether the sera show only a slightly different specificity

Seven out of 9 strains belonging to the new O group 14 (a homologous titre of 6400–12800) gave heterologous reactions with some of *Habs* O groups Cross reactions with O groups 3 and 6 O groups 3 and 7 O groups 6 and 7 O groups 7 and 8 and O groups 7 and 9 have thus been observed On the other hand some strains which belonged to O groups 2 3 5 6 9 and 11 gave heterologous reactions with O group 14 (a titre of 200–800) The numerous cross reactions might possibly be explained by the presence of a thermostable surface antigen differing from the other O antigens but may also be explained simply by a lower specificity of the high titered O serum

The frequency of O groups in this investigation i.e. 58 per cent of the strains belonging to O groups 2 3 5 2/5, 6 and 11, is the same as in other comparable studies (14)

Strains belonging to the same O group have been isolated several times from the same sort of material from the same patient thus indicating that the O group determination could possibly be used for epidemiological investigations

Only few strains were not isolated from human subjects 25 per cent of strains from surface water belonged to O group 3 O group 3 strains were also numerous in samples from stools (2 of 9 strains from persons outside a hospital environment 13 per cent of strains from hospitalized patients), which might indicate a faecal contamination of the water examined *Gregacs et al* draw the same conclusion from a comparison between 139 strains from water and 326 from faeces (3) *Janssen & Meyers* found precipitating antibodies against *Ps aeruginosa* among up to 6 per cent of fish from rivers in heavily populated areas but no antibodies among fish from sparsely populated areas (6) *Thörne* found that the number of *Ps aeruginosa* from water in lakes is increased with a rising content of *E coli* (19)

Thirty one per cent of the strains isolated from animals belonged to O group 4 an O group which occurs very seldom in this material In larger materials of strains from animals other O groups are predominant Thus in cases of inflammation of the udder O groups 6 and 13 (15) and O groups 6 13 and 3 (18) predominate while from bull sperm O groups 3 1 2/5 and 2 (23) and from faeces from four different species of animals O group 13 (20) predominate Thus, there seems to be a difference in the distribution among animals and human subjects

Fourty three per cent of strains isolated

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ISOLATION OF PLASMA MEMBRANE FRAGMENTS FROM BHK 21 CELLS

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We have isolated fractions rich in plasma membranes from baby hamster kidney cells (BHK21) by using a modification of the method of *Wallach & Kamat* (1966) The specific activity of Na K activated ATPase was 10–20 fold higher in the plasma membranes than in the original homogenate Antiserum against whole cells gave a strong plasma membrane fluorescence and agglutinated intact BHK21 cells Quantitative agglutination inhibition studies indicated a 20 fold concentration of the plasma membrane antigens in the isolated plasma membrane fraction In isolated plasma membrane fractions NADH diaphorase activity was low and succinate dehydrogenase activity not detectable

Plasma membranes have been isolated from various mammalian nucleated cells rat liver (*Neville* 1960 *Emmelot et al* 1964 *Takeuchi & Terayama* 1965 *Coleman et al* 1967) mouse liver (*Herzenberg & Herzenberg* 1961) Ehrlich ascites carcinoma cells (*Wallach & Kamat* 1966) L cells (*Warren et al* 1966) and HeLa cells (*Bosmann et al* 1968)

Methods that are suitable for one type of cell may not isolate plasma membranes satisfactorily from other types Therefore in each case some means of recognizing the plasma membranes is necessary (*Boyle* 1967) Enzyme analysis is usually used to distinguish the plasma membranes from the smooth cytomembranes The most specific enzymes for marking the plasma membrane fractions seem to be the 5 nucleotidase (*Emmelot et al* 1964 *Coleman et al* 1967 *Song & Bodansky* 1967) and Na K activated ATPase (*Emmelot et al* 1964 *Wallach & Kamat* 1966) Adenyl cyclase is probably also mainly found in the plasma membranes (*Manarth et al* 1969) NADH diaphorase (*Wallach & Kamat* 1966, *Dallner et al* 1966) and glucose 6 phosphatase (*Emmelot et al* 1964 *Coleman et al* 1967 *Song & Bodansky* 1967) are confined to the cytomembranes in nucleated cells In addition the purity of the plasma membrane fractions have been checked by other methods such as electron microscopy chemical analysis and immunological studies

We are studying the alterations in the plasma membrane caused by Semliki Forest virus a group A arbovirus This virus leaves the host cell by budding through the plasma membrane (*Acheson & Tamm* 1967) The cells we use are the BHK21 line fibroblasts derived from hamster kidney (*MacPherson & Stoker* 1962) which can be grown in continuous culture *Klenk & Choppin* (1969) have isolated BHK21 plasma membranes for lipid analyses They used the method of *Warren et al* (1966) The plasma membranes

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were denatured by fluorescein mercuric acetate and their purity could be checked only by phase and electron microscopy

We first tried the methods of *Coleman et al* (1967) and *Bosmann et al* (1968) for separating plasma membranes from BHK21 cells (*Gahmberg & Simons* 1969a *Gahmberg & Simons* 1969b) However although plasma membrane marker enzymes were concentrated both methods gave very variable results in our hands Here we report the results obtained for BHK21 cells by a modification of the method of *Wallach & Kamat* (1966) This method gave reproducible results In the plasma membrane fraction the Na K activated ATPase and plasma membrane antigens were concentrated about 20 fold

MATERIALS AND METHODS

Cells

Baby hamster kidney cells (BHK21) line WI 2 (*Vaheri et al* 1965) kindly supplied by Dr A Vaheri were grown in Roux bottles for 3-4 days in 40 ml BHK21 tissue culture medium (*MacPherson & Stoker* 1962)

Chemical Determinations

Protein was measured by the method of *Lowry et al* (1951) with bovine serum albumin as standard The reaction mixtures contained 0 1 per cent [illegible] Phosphorus was assayed by the method of *Ame* (1966)

Enzymes

The enzymes were assayed by the methods of *Wallach & Kamat* (1966)—ATPase and NADH diaphorase *Song & Bodansky* (1967)—5 nucleotidase with AMP as substrate *Allison & Sandelin* (1963)—acid *β* glycerophosphatase and *β* glucuronidase *King* (1967)—succinate dehydrogenase The reaction mixtures for the phosphatases were as described but the incubation volumes were reduced to 0 3 ml

Immunization Procedure

BHK21 cells were washed three times and about 0 05 g of packed cells (wet weight) was suspended in 1 ml of 0 3 M sucrose pH 7 4 (adjusted with $NaHCO_3$) This was added to 1 ml of Freund s complete adjuvant Each rabbit was immunized every third week with this mixture Immunization was continued for 9 weeks and the rabbits were bled 10 days after the last injection The sera were stored at —40

Immunofluorescent Staining

BHK21 cells were grown on microscope slides in Roux bottles The slides were washed three times with 0 15 M NaCl 0 005 M Tris pH 7 4 and fixed with 40 per cent acetone – 7 per cent formaldehyde – 53 per cent H_2O for 30 seconds Then antiserum or antiserum absorbed as described below diluted 1 100 with 0 15 M NaCl 0 005 M Tris pH 7 4 was added and the cells were incubated at 37 for 30 min The cells were washed with NaCl Tris and a few drops of fluorescein labelled sheep anti rabbit gamma globulin (fluorescein/protein molar ratio 1 5 kindly supplied by Dr E Linder of this department) was added and the slides incubated at 37 for 2 h and washed with NaCl Tris A Wild fluorescence microscope was used to examine the slides and for photographing with excitation filter UG 1 (1 mm) and UV absorbing barrier filter GG 9

Absorption of Antisera and Agglutination of Cells

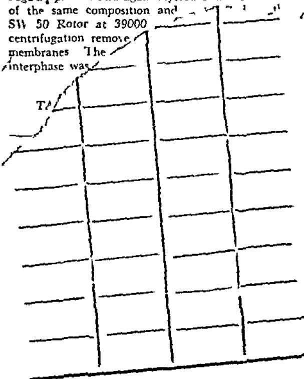
For this we used antiserum (0 6 ml) against whole BHK21 cells which agglutinated 0 2 ml (1 mg protein) of intact BHK21 cells at a dilution of 1 320 This antiserum was diluted 1 100 and absorbed with varying amounts of the subcellular fractions by gently shaking at 4 for 24 h The samples were centrifuged in a Christ UJ1 centrifuge (table centrifuge) at full speed for 2 h and the supernatants recovered

BHK21 cells were washed three times and suspended in 0 15 M NaCl 0 005 M Tris pH 7 4 The cell suspension (0 2 ml containing 1 mg protein) was added to absorbed or unabsorbed antiserum (0 6 ml) The tubes were allowed to stand at room temperature for 3-4 h and then shaken by hand A plus reaction was defined as the formation of cell clumps that were not dissociated by shaking

Isolation Procedure

The cells were scraped from the Roux bottles with a rubber policeman and taken up in 0 25 M sucrose 0 005 M Tris 0 0002 M $MgSO_4$ pH 7 4 About 10 g of packed cells was used for each preparation The *cell suspension* was centrifuged in a Sorvall SS 1 centrifuge at 4000 rev/ min for min and washed twice with the same cells were the suspended in 60 ml buffer and pla d in a pressure homo was a simplified version of that descri *te & Commerford* (1961) The cells brated for 20 min under 60-70 kg/c pressure and cat uously released thro tube (diameter 1 mm) The Mg io for preventing nuclear rupture (*H merford* 1961 *Attt* 1969) After the homogenate was made 0 001 to EDTA and cen ed in the

at 12500 rev/min (13600 × g) for 15 min All g values refer to the middle of the centrifuge tubes The supernatant was saved The pellet was suspended in 60 ml of the same buffer and EDTA was added to a concentration of 0 001 M This suspension was then centrifuged at 12500 rev./min for 15 min to give the nuclear pellet containing nuclei mitochondria and lysosomes The super natant was combined with the previous super natant and these were centrifuged in the same rotor at 25000 rev/min (54500 × g) for 90 min The supernatant was called the soluble fraction The pellet from this centrifugation was suspended in about 60 ml 0 01 M Tris pH 8 6 by a few strokes with a Dounce homogenizer (Kontes Glass Co Vineland N J) and centrifuged at 25000 rev/min for 90 min The pellet was suspended in 0 001 M Tris pH 8 6 with the Dounce homogenizer and again centrifuged at 25000 rev./min for 90 min The pellet constituted the *total microsomal fraction* This was suspended in 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 and dialysed against the same buffer for 2 h Two ml of the dialysed mem brane suspension was layered over 3 ml Ficoll solution (Pharmacia) of density 1 050 at 20 The Ficoll was prepared according to *Wallach & Kamat* (1966) and contained 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 Centrifugation in the Spinco SW 50 Rotor at 39000 rev/min (125000 × g) for 5 h produced two main fractions a white band at the Ficoll buffer interphase and a yellow aggregat ed pellet The pellet was saved The white band *was diluted about 1 5 with 0 001 M Tris* 0 001 M $MgSO_4$ pH 8 6 and again layered over 3 ml Ficoll of the same composition and ... SW 50 Rotor at 39000 ... centrifugation remove... membranes The ... interphase was...



from the first Ficoll centrifugation were each di luted with 0 001 M Tris pH 8 6 and centrifuged in the Spinco SW 50 Rotor at 39000 rev./min for 45 min The pellets were finally suspended in 0 001 M Tris pH 8 6 The fraction from the Ficoll buffer interphase was shown to contain the *plasma membranes* and called *F1* the washed pellet from the first Ficoll centrifugation contained most of the *endoplasmic membranes* and was called F2

RESULTS

The protein distribution of one representative experiment was homogenate 330 mg nuc lear pellet 170 mg soluble fraction 100 mg the combined Tris washings 35 mg total mi crosomal fraction 10 mg, F1 1 5 mg and F2 8 mg

The enzyme activities of fraction F1 (plas ma membranes) in 3 different experiments are shown in Table 1 The activity of the Na K⁺ activated ATPase was high, the spe cific activity had increased 10–20 fold over the homogenate The activities of 5 nucleo tidase and acid β glycerophosphatase were also increased when compared with the homogenate No succinate dehydrogenase ac tivity was detectable The β glucuronidase activity was quite low

The enzymes of fraction F2 (endoplasmic ... *ian et al* 1967, *Song & Bodansky* 1967) ... onfined to the cytomembranes in nuc ... cells In addition the purity of the ... membrane fractions have been check ... other methods such as electron mi ...y, chemical analysis and immunologi ...lies

...are studying the alterations in the ... membrane caused by Semliki Forest ... group A arbovirus This virus leaves ... cell by budding through the plasma ... (*Acheson & Tamm* 1967) The ...se are the BHK21 line fibroblasts ...om hamster kidney (*MacPherson* ...1962) which can be grown in con ...lture *Klenk & Choppin* (1969) ...d BHK21 plasma membranes for ... They used the method of *War* ... (1966) The plasma membranes

TABLE 2 *Enzyme Activities of Fraction F2 (Endoplasmic Reticulum) Three Different Experiments Are Shown*

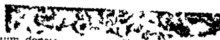
	Specific activity			Relative specific activity (F2/homogenate)		
	1	2	3	1	2	3
Na K ATPase	1 33	2 40	1 18	1 39	2 65	2 20
5 nucleotidase	0 91	0 56	0 46	1 30	1 17	1 14
Acid phosphatase	1 75	0 80	0 99	1 37	1 15	1 54
Beta glucuronidase	1 33	0 73	1 07	1 71	1 59	1 95
Succinate dehydrogenase	9 6	14 4	4 1	0 74	1 10	0 33
NADH diaphorase	1 84	2 02	3 15	2 50	3 38	4 06

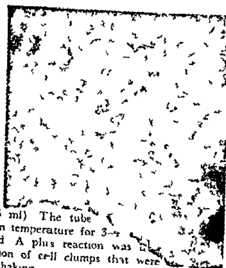
Enzyme units as in Table 1

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Immunofluorescence

Antiserum against BHK21 cells gave a strong ring reaction (Fig 1) with whole





sodium dodecyl ... en ... Phosphorus was assayed by the method of *Ames* (1966)

Enzymes

The enzymes were assayed by the methods of *Wallach & Kamat* (1966)—ATPase and NADH diaphorase *Song & Bodansky* (1967)—5 nucleotidase with AMP as substrate *Allison & Sandlin* (1963)—acid β glycerophosphatase and β glucuronidase *King* (1967)—succinate dehydrogenase The reaction mixtures for the phosphatases were as described but the incubation volumes were reduced to 0 3 ml

Immunization Procedure

BHK21 cells were washed three times and about 0 05 g of packed cells (wet weight) was suspended in 1 ml of 0 3 M sucrose pH 7 4 (adjusted with $NaHCO_3$) This was added to 1 ml of Freund's complete adjuvant Each rabbit was immunized every third week with this mixture Immunization was continued for 9 weeks and the rabbits were bled 10 days after the last injection The sera were stored at —40

(0 6 ml) The tube ... room temperature for 3–... hand A plus reaction was ... mation of cell clumps that were ... by shaking

... ane fluor ... with iso ... whole ... mem

Isolation Procedure

The cells were scraped from the Roux ... with a rubber policeman and taken up in 0 2 ... to sucrose 0 005 M Tris 0 0002 M $MgSO_4$ pH 7 4 About 10 g of packed cells was used for each preparation The cell suspension was centrifuged in a Sorvall SS 1 centrifuge at 4000 rev /min for 1 min and washed twice with the same buffer The cells were then suspended in 60 ml of the same buffer and placed in a pressure homogenizer This was a simplified version of that described by *Hunter & Commerford* (1961) The cells were equilibrated for 20 min under 60–70 kg/cm of nitrogen pressure and cautiously released through the outlet tube (diameter ? mm) The Mg ions are essential for preventing nuclear rupture (*Hunter & Commerford* 1961 ... 1969) After homogenization the homogenate was made 0 001 M with respect to EDTA and centrifuged in the Spinco 30 Rotor

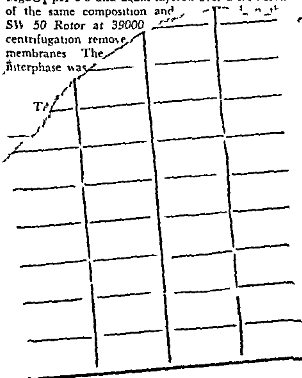
at 12500 rev./min (13600 × g) for 15 min All g values refer to the middle of the centrifuge tubes The supernatant was saved The pellet was suspended in 60 ml of the same buffer and EDTA was added to a concentration of 0 001 M This suspension was then centrifuged at 12500 rev./min for 15 min to give the nuclear pellet containing nuclei mitochondria and lysosomes The super natant was combined with the previous super natant and these were centrifuged in the same rotor at 25000 rev /min (54500 × g) for 90 min The supernatant was called the soluble fraction The pellet from this centrifugation was suspended in about 60 ml 0 01 M Tris pH 8 6 by a few strokes with a Dounce homogenizer (Kontes Glass Co Vineland N J) and centrifuged at 25000 rev /min for 90 min The pellet was suspended in 0 001 M Tris pH 8 6 with the Dounce homogenizer and again centrifuged at 25000 rev /min for 90 min The pellet constituted the *total microsomal fraction* This was suspended in 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 and dialysed against the same buffer for 2 h Two ml of the dialysed mem brane suspension was layered over 3 ml Ficoll solution (Pharmacia) of density 1 050 at 20 The Ficoll was prepared according to *Wallach & Kamat* (1966) and contained 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 Centrifugation in the Spinco SW 50 Rotor at 39000 rev /min (125000 × g) for 5 h produced two main fractions a white band at the Ficoll buffer interphase and a yellow aggregat ed pellet The pellet was saved The white band was diluted about 1 5 with 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 and again layered over 3 ml Ficoll of the same composition and

SW 50 Rotor at 39000

centrifugation remove

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interphase was



from the first Ficoll centrifugation were each di luted with 0 001 M Tris pH 8 6 and centrifuged in the Spinco SW 50 Rotor at 39000 rev./min for 45 min The pellets were finally suspended in 0 001 M Tris pH 8 6 The fraction from the Ficoll buffer interphase was shown to contain the *plasma membranes* and called *F1* the washed pellet from the first Ficoll centrifugation contained most of the *endoplasmic membranes* and was called *F2*

RESULTS

The protein distribution of one representative experiment was homogenate 330 mg nuc lear pellet 170 mg soluble fraction 100 mg the combined Tris washings 35 mg total mi crosomal fraction 10 mg, F1 1 5 mg and F2 8 mg

The enzyme activities of fraction F1 (plas ma membranes) in 3 different experiments are shown in Table 1 The activity of the Na K activated ATPase was high the spe cific activity had increased 10–20 fold over the homogenate The activities of 5 nucleo tidase and acid β glycerophosphatase were also increased when compared with the homogenate No succinate dehydrogenase ac tivity was detectable The β glucuronidase activity was quite low

The enzymes of fraction F2 (endoplasmic

...an et al 1967, Song & Bodansky 1967)

...onfined to the cytomembranes in nuc

... cells In addition the purity of the

... membrane fractions have been check

... other methods such as electron mi

...y chemical analysis and immunologi

...ies

...are studying the alterations in the

...membrane caused by Semliki Forest

...group A arbovirus This virus leaves

...cell by budding through the plasma

... (*Acheson & Tamm* 1967) The

... e are the BHK21 line fibroblasts

...om hamster kidney (*MacPherson*

...1962) which can be grown in con

...lture *Klenk & Choppin* (1969)

...d BHK21 plasma membranes for

...s They used the method of *War*

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TABLE 2 *En yme Actıvıtıes of Fractıon F2 (Endoplasmıc Retıculum) Three Dıfferent Experıments Are Shoun*

	Specıfic actıvıty			Relatıve specıfic actıvıty (F2/homogenate)		
	1	2	3	1	2	3
Na K ATPase	1 33	? 40	1 18	1 39	2 65	2 20
5 nucleotıdase	0 91	0 56	0 46	1 30	1 17	1 14
Acıd phosphatase	1 75	0 80	0 99	1 37	1 15	1 54
Beta glucuronıdase	1 33	0 73	1 07	1 71	1 59	1 95
Succınate dehydrogenase	9 6	14 4	4 1	0 74	1 10	0 33
NADH dıaphorase	1 84	? 02	3 15	2 50	3 38	4 06

Enzyme unıts as ın Table 1

tıvated ATPase and 5 nucleotıdase were low The actıvıty of acıd β glycerophosphatase was low but the actıvıty of β glucuronıdase was hıgher than ın the homogenate Succınate dehydrogenase actıvıty varıed ın strength but was always present

Immunofluorescence

Antıserum agaınst BHK21 cells gave a strong rıng reactıon (Fıg 1) wıth whole



...nce Rabbıts ... cells ın ... esultıng ... mı ... ep



Fıg 2 Inhıbıtıon of plasma membrane fluorescence by absorptıon of the antıserum wıth ısolated plasma membranes Antıserum agaınst whole BHK21 cells was ıncubated wıth the plasma membrane fractıon at 4 C for 24 h and then added to cells grown on mıcroscope slıdes After washıng fluorescent sheep antı rabbıt gamma globulın was applıed Magnıfıcatıon 600 ×

cells whıch *Moller* (1961) descrıbed as characterıstıc of plasma membranes When the antıserum was absorbed wıth the plasma membrane fractıon (F1) the plasma membrane fluorescence dısappeared but a faınt cytoplasmıc fluorescence remaıned (Fıg 2) Exact quantıtatıon of the ımmunofluorescence

at 12500 rev/min (13600 × g) for 15 min All g values refer to the middle of the centrifuge tube The supernatant was saved The pellet was suspended in 60 ml of the same buffer and EDTA was added to a concentration of 0 001 M This suspension was then centrifuged at 12500 rev/min for 15 min to give the nuclear pellet containing nuclei mitochondria and lysosomes The supernatant was combined with the previous supernatant and these were centrifuged in the same rotor at 25000 rev/min (54500 × g) for 90 min The supernatant was called the soluble fraction The pellet from this centrifugation was suspended in about 60 ml 0 01 M Tris pH 8 6 by a few strokes with a Dounce homogenizer (Kontes Glass Co Vineland N J) and centrifuged at 25000 rev/min for 90 min The pellet was suspended in 0 001 M Tris pH 8 6 with the Dounce homogenizer and again centrifuged at 25000 rev/min for 90 min The pellet constituted the *total microsomal fraction* This was suspended in 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 and dialysed against the same buffer for 2 h Two ml of the dialysed membrane suspension was layered over 3 ml Ficoll solution (Pharmacia) of density 1 050 at 20 The Ficoll was prepared according to *Wallach & Kamat* (1966) and contained 0 001 M Tris 0 001 M $MgSO_4$ pH 8 6 Centrifugation in the Spinco SW 50 Rotor at 39000 rev/min (145000 × g) for 3 h produced two main fractions a white band at the Ficoll buffer interphase and a yellow aggregated pellet The pellet was saved The white band was diluted about 1 5 with 0.001 M Tris 0.001 M $MgSO_4$ pH 8 6 and again layered over 3 ml Ficoll of the same composition and centrifuged in the SW 50 Rotor at 39000 rev/min for 5 h This centrifugation removed some trapped endoplasmic membranes. The band from the Ficoll buffer interphase was collected This band and the pellet

from the first Ficoll centrifugation were each diluted with 0 001 M Tris pH 8 6 and centrifuged in the Spinco SW 50 Rotor at 39000 rev/min for 45 min. The pellets were finally suspended in 0 001 M Tris pH 8 6 The fraction from the Ficoll buffer interphase was shown to contain the *plasma membranes* and called *F1* the washed pellet from the first Ficoll centrifugation contained most of the *endoplasmic membranes* and was called *F2*

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The protein distribution of one representative experiment was homogenate 330 mg nuclear pellet 170 mg soluble fraction 100 mg the combined Tris washings 35 mg total microsomal fraction 10 mg F1 1 5 mg and F2 8 mg

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The enzyme activities of fraction F2 (endoplasmic membranes) are shown in Table 2 The specific activity of NADH diaphorase was relatively high The activities of Na+ K+ ac

TABLE 1 *Enzyme Activities of Fraction F1 (Plasma Membranes) Three Different Experiments Are Shown*

	Specific activity			Relative specific activity (F1/homogenate)		
	1	2	3	1	2	3
ATPase	8 26	12 1	12 7	8 60	13 4	23 8
[illegible]ase	1 09	0 75	0 91	1 57	1 55	2 27
[illegible] phosphatase *	3 73	2 20	3 13	2 95	3 16	4 90
[illegible]uronidase†	0 37	0 42	0 62	0 47	0 91	1 12
Succinate dehydrogenase§	0	0	0	[illegible]	0	0
NADH-diaphorase§	0 43	0 45	0 95	0 59	0 75	1 23

* μmoles Pi liberated/mg protein/h/37

† μmoles substrate utilized/mg protein/h/37

§ μmole substrate utilized/mg protein/min/20

TABLE 2 *Enzyme Activities of Fraction F2 (Endoplasmic Reticulum) Three Different Experiments Are Shown*

	Specific activity			Relative specific activity (F2/homogenate)		
	1	2	3	1	2	3
Na K ATPase	1 33	2 40	1 18	1 39	2 65	2 20
5 nucleotidase	0 91	0 56	0 46	1 30	1 17	1 14
Acid phosphata e	1 75	0 80	0 99	1 37	1 15	1 54
Beta glucuronidase	1 33	0 73	1 07	1 71	1 59	1 95
Succinate dehydrogenase	9 6	14 4	4 1	0 74	1 10	0 33
NADH diaphorase	1 84	2 02	3 15	2 50	3 38	4 06

Enzyme units as in Table 1

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Antiserum against BHK21 cells gave a strong ring reaction (Fig 1) with whole

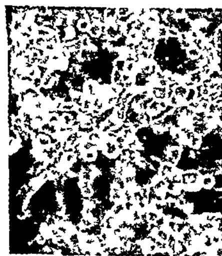


Fig 1 Plasma membrane fluorescence Rabbits were immunized with whole BKH21 cells in Freund s complete adjuvant and the resulting antiserum was incubated with cells grown on microscope slides After washing fluorescent sheep anti rabbit gamma globulin was applied Magnification 600 ×



Fig 2 Inhibition of plasma membrane fluorescence by absorption of the antiserum with isolated plasma membranes Antiserum against whole BHK21 cells was incubated with the plasma membrane fraction at 4 C for 24 h and then added to cells grown on microscope slides After washing fluorescent sheep anti rabbit gamma globulin was applied Magnification 600 ×

cells which *Moller* (1961) described as characteristic of plasma membranes When the antiserum was absorbed with the plasma membrane fraction (F1) the plasma membrane fluorescence disappeared but a faint cytoplasmic fluorescence remained (Fig 2) Exact quantitation of the immunofluorescence was not possible (see however *Collier* 1968)

sponse should be expected to act against only one larval stage (*Soulsby* 1961)

The significant difference between the number of injected larvae which managed to develop beyond the second stage in immune mice and controls (Table 1) further supports the view that larvae are attacked in the lungs in immune mice whatever the mechanism may be Furthermore, it is impressive that so few larvae developed in the controls which indicates that second stage larvae are capable of only a slight development when injected intravenously

In spite of the arbitrary limit (500 μ) of second stage larvae (*Soulsby* 1961) also the determination of the larval stages gave interesting information concerning the larval migration (Table 1) The sudden occurrence of third stage larvae in orally challenged controls, and their high percentage of the total number of larvae must be due to a sudden migration of these larvae to the lungs rather than to a progressive development from second stage larvae already present in the lungs It is in support of *Soulsby*'s view (1961) that most of the second stage larvae reaching the liver will moult at about the 4th to 5th day after the infection, and that the lungs will be reached by these larvae Histological examinations of livers from infected mice have shown that the majority of the larvae detected from the 4th day was apparently third stage larvae (*Bindseil* 1969b)

The fact that only 4 1 per cent of the larvae detected from the 7th to 10th day in controls challenged intravenously were third to fourth stage larvae whereas 95 5 per cent of the larvae recovered in almost the same period in controls challenged by oral route were third to fourth stage larvae seems to be strong evidence of the preceding suggestion that the large majority of the third to fourth stage larvae in the lungs is not developed from second stage larvae already present in the lungs

It is difficult to explain why haemorrhages on the pleural surface of the lungs were detected almost exclusively in controls challenged by the oral route and not in those challenged intravenously The reason may be that the lungs of the former group are reached by third stage larvae which are 2–3 times the size of second stage larvae reaching the lungs of animals in the latter group However in the work by *Arean* & *Crandall* (1962) haemorrhages were observed in the lungs of rabbits injected with second stage larvae

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TABLE 2 *Number of Non Hatched Eggs and Free Larvae of* Ascaris Suum *in the Caecal Contents of Mice 8 Hrs after Challenge with 3 000* Ascaris Suum *Eggs together with the Number of Liver Larvae at the same Time*

Group of mice	Mouse no	Caecal contents: No of eggs	Caecal contents: Free larvae	Liver: No of larvae	Mean no of liver larvae
	1	0	c 5	592	
	2	0	c 10	930	
	3	0	c 5	1164	
	4	0	0	642	
Controls	5	c 5	c 15	498	855
	6	0	0	1026	
	7	0	0	1072	
	8	0	c 5	618	
	9	0	0	1206	
	10	0	c 5	798	
	11	c 10	c 10	324	
	12	c 75	c 50	308	
	13	0	c 150	542	
	14	c 200	c 80	96	
Immune mice	15	c 80	0	398	325
	16	0	0	248	
	17	c 80	0	176	
	18	c 80	c 40	136	
	19	0	c 40	768	
	20	0	0	258	

of the controls until the 5th day Taking into consideration this observation and also the [illegible] difference between the number of lung [illegible]ae in immune mice and controls infected [illegible] with eggs it is obvious that the difference in the larval counts is due to an anti larval activity in the gut accordingly as the challenge is oral or intravenous as the liver apparently does not participate in the defence mechanism (*Bindseil* 1969b)

The results of Experiment 2 (Table 2) strongly support the preceding view

The difference between the number of eggs and larvae in the caecal contents in immune mice and controls strongly indicates that the protective immunity is already working in the gut lumen itself by influencing the process of [illegible]ing and [illegible]n tration of the [illegible] muc[illegible] the larv[illegible] observations [illegible] port [illegible] that the [illegible] immune mice is [illegible] number of la[illegible]

vae starting migration and that the penetration into the gut wall is delayed (*Bindseil* 1969b)

Furthermore Experiment 1 in the present study seems to have provided additional knowledge about the larval migration in non immune and immune mice as regards the lung stages

The sudden fall in the number of lung larvae after the 5th day in immune mice challenged intravenously with second stage larvae may be connected with the conspicuous anti larval activity seen in lungs of immune mice challenged orally (*Bindseil* 1969b) In the latter experiment it was a reaction against third to fourth stage larvae and it was suggested that the reaction might be either non specific or specific in nature Provided that the mechanism is the same in the 2 studies it seems more likely that it is non specific due to physico-chemical changes in heavily inflamed tissue, as a specific protective re

sponse should be expected to act against only one larval stage (*Soulsby* 1961)

The significant difference between the number of injected larvae which managed to develop beyond the second stage in immune mice and controls (Table 1) further supports the view that larvae are attacked in the lungs in immune mice whatever the mechanism may be Furthermore it is impressive that so few larvae developed in the controls, which indicates that second stage larvae are capable of only a slight development when injected intravenously

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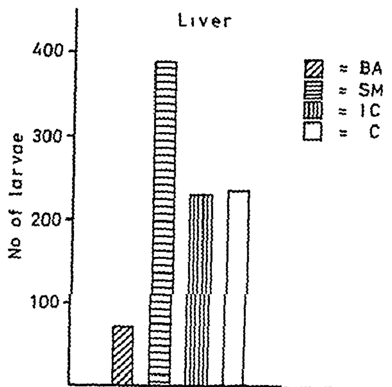


Fig 1 Mean number of *Ascaris suum* larvae in the liver 2 days after challenge of mice previously immunized with bovine albumin (BA) infected with *Schistosoma mansoni* (SM) and injected with indigo carmine (IC) *Controls (C)* Average of 8 9 11 and 10 mice respectively

mour Pharmaceutical Comp England) was dissolved in saline to make a 0 5 per cent solution The BA solution was administered intraperitoneally with Freund's complete adjuvant (Difco Lab USA) in a ratio of 1 to 1 or without any adjuvant

Schistosoma mansoni infection An Egyptian strain of *Schistosoma mansoni* was obtained from Dr C C Cridland (The Danish Bilharziasis Lab) who also carried out the percutaneous infection of the mice At necropsy the infection was verified by histology of the liver

Indigo carmine Indigo carmine was suspended in saline and concentrations of 1 ½ and ¼ per cent were made The suspension was injected intraperitoneally

Infective eggs of Ascaris suum The preparation of infective *Ascaris suum* eggs and their administration to the mice were carried out as described elsewhere (Bindseil 1969a)

Post mortem examination The larval counts in the livers were done as described elsewhere (Bindseil 1969b)

EXPERIMENTS

The mice were divided into groups of equal size Three of the groups were used for Experiments 1–3 and the 4th group served as the control group All the mice were of almost the same age at the time of challenge with *Ascaris suum* Prior to challenge the mice were treated as follows

Experiment 1 The mice were subjected to 3 injections of 0 25 ml of the mixture of BA and adjuvant at intervals of 2 or 3 days and 8 days later they were injected with 0 25 ml of BA only Seven days after the last injection the mice were challenged with 2 000 *Ascaris suum* eggs The mice were killed on the 2nd day following infection and the livers were examined for larvae

Experiment 2 The mice were infected percutaneously with approximately 200 cercariae of *Schistosoma mansoni* and challenged 78 days later with 2 000 *Ascaris suum* eggs The mice were killed and examined as in the preceding experiment

Experiment 3 The mice were given 4 injections of 0 25 ml of the indigo carmine suspension at intervals of 7 days between the first 3 injections and 14 days between the 3rd and the 4th injection The concentrations used of indigo carmine were 1 ¼ ½ and ½ per cent respectively The mice were challenged with 2 000 *Ascaris suum* eggs the day after the 4th injection and they were sacrificed 2 days later and larval counts were made in the livers

The controls (the 4th group) received an infection with 2 000 *Ascaris suum* eggs they were killed 2 days later and the livers were examined for larvae

TABLE 1 *Effect of Different Stimulations upon Challenge of Mice with Ascaris Suum*

Primary stimulation	Ascaris Suum challenge dose (eggs/mouse)	No of mice	Larval counts in liver Mean (range)
Immunization with BA	2 000	8	70 (10–144)
Infection with SM	2 000	9	388 (114–670)
Injection with IC	2 000	11	228 (36–702)
None (controls)	2 000	10	234 (72–596)

The Mice were sacrificed 2 days after challenge and the livers examined for larvae
BA = Bovine albumin SM = *Schistosoma mansoni* IC = Indigo carmine

RESULTS

It is seen in Fig 1 and Table 1 that in mice previously immunized with BA only a small number of *Ascaris suum* larvae was recovered from the liver 2 days after the challenge The mean number of larvae recovered in that group was 70 compared to 234 in the controls A t test was not carried out as the F value exceeded the 95 per cent level of F

Furthermore it is shown that more larvae were detected on the same day in the livers of mice infected previously with *Schistosoma mansoni* than in the controls The difference between the means was not found to be statistically significant but the observed difference indicates a tendency towards an effect caused by the schistosomes as the t value corresponded to about 93 per cent probability

Repeated injections of indigo carmine prior to the *Ascaris suum* infection had apparently no influence on the mean larval count compared to that of the controls as the former was 228 and latter 234 A t test was not applied

DISCUSSION

It seems that the immunization of the mice with BA (plus *Freund's* adjuvant) and the infection with *Schistosoma mansoni* have interfered with the course of the *Ascaris suum* infection Thus the immunization with BA gave an increased resistance and the previous infection with the schistosomes possibly a decreased resistance against the infection as determined by larval counts in the liver 2 days after the challenge with the ascarids

The present investigation gives no explanation of the mechanisms underlying the enhanced resistance caused by the injections with BA First of all the increased resistance might be due to antigenic cross reactions between BA and *Ascaris suum* larvae as regards antigens functional in immunity but it seems more reasonable to believe that the mechanisms are to be defined according to the following explanations though other possibilities may exist

It may be suggested that the increased amount of immunoglobulins following the immunization somehow unspecifically influences the *Ascaris suum* larvae and their environment in a deleterious way It could also be suggested that the repeated injections of BA have stimulated the antibody forming mechanisms comparable to that sometimes observed following injections of apparently inert materials (e g *Anzueiler* 1959)

The enhanced resistance may be related for instance to a proliferation of cells in RES and particularly of those responsible for antibody production as seen by *Pernis & Paronetto* (1962) who demonstrated an adjuvant effect of silica on antibody production Furthermore the reaction may be associated with an increased number of RES cells attributable to a stimulation of dormant cells developing to active RES-cells rather than to a proliferation of existing RES-cells (*Howard et al* 1958)

Considering the above stated features an enhanced resistance in the mice injected with

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EXPERIMENTAL ASCENDING SPHEROPLAST INFECTIONS OF THE URINARY TRACT

Some Factors Affecting the Viability of Spheroplasts within the Urinary Tract

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The influence of urine osmolality and urinary pH on the survival of spheroplasts from *P vulgaris* was studied in ascending urinary tract infections in rabbits treated with penicillin Spheroplasts were isolated from the urine of three out of fourteen rabbits with hypotonic urines All three had a urinary pH below 5 5 No spheroplasts were isolated from the hypotonic urines with pH above 5 5 of the other eleven rabbits However in this latter group spheroplasts were isolated from the kidney tissue of four rabbits In the control group consisting of animals spheroplasts were isolated in the urine and in the kidney tissue of 19 and 16 rabbits respectively These findings were discussed with regard to urine osmolality urinary pH and treatment of urinary tract infections with penicillin

An increasing number of reports have been published during the last few years on the isolation of spheroplasts and L forms from different clinical sources (*Nattelle & Depa* 1960 *Braude et al* 1961, *Kagan* et al 19 *Lapinski & Flakes* 1967 *Mattman* 1968 *Chatman et al* 1969) in particular from the urine of patients with symptoms of urinary tract infection when ordinary cultures have yielded no growth (*Braude et al* 1961 *Gutman et al* 1965 *Gutman et al* 1967, *Kristianson & Guze* 1968 *Conner et al* 19) Some workers have found spheroplasts and L forms to persist in the urinary tract during treatment with certain antibiotics When the treatment ceased the spheroplasts reverted to bacterial phase and an aggravation of the clinical symptoms occurred (*Braude et al* 1961 *Gutman et al* 196 *Gutman et al* 196) As urine and kidney tissue especially medulla and the papillae are hypertonic compared to other organs and body fluids the urinary tract offers conditions for the survival of osmotically fragile organisms

This work was undertaken in order to study the influence of urine osmolality and pH on the survival and persistence of spheroplasts in the urinary tract as an earlier *in vitro* study suggested that spheroplasts were viable and multiplying in acid hypotonic media (*Gnarpe & Edebo* to be published)

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MATERIALS AND METHODS

Bacteria A strain of *Proteus vulgaris* X 19 obtained from the type culture collection of the National Bacteriological Laboratory Stockholm was used as infecting organism

Cultivation Blood agar (Difco) endo agar (Difco) and sodium-desoxycholate-citrate agar (Difco) were used for cultivation of ordinary bacteria from urine A medium (CA medium) similar to that described by *Lederberg & St Clair* (1958) was used for cultivation of spheroplasts Casamino acids 10 0 g NaCl 3 5 g glucose 1 0 g sucrose 0 3 M and distilled water ad 1000 For solid media 10 0 agar was added

After autoclaving 10 ml 20 per cent $MgSO_4$ was added before the plates were poured The urine samples were serially diluted in a hypo tonic diluent (0 01 M phosphate buffer pH 7 0) and in the CA medium without agar in order to isolate osmotically stable and fragile organisms respectively

Thin sections of the kidneys were cut and immersed into nutrient broth and into fluid CA medium for isolation of bacteria and spheroplasts respectively

Determination of urine conductivity and pH The conductivity of different urine specimens was measured with the aid of a direct reading onductivity measuring bridge (Philips Model PR 9501) Since urea did not protect against osmotic lysis (*Gnarpe* & *Edebo* to be published) the conductivity was used instead of osmolality for estimating the osmotically pro tective activity of urine According to earlier investigations (*Gnarpe* & *Edebo* to be published) the critical value for the osmolality amounts to around 400 mosm/l with regard to th pre ervation of spheroplasts induc d from gram negative bacteria

The pH was measured with a pH meter (Ti trator Type TTT1 Radiometer Copenhagen)

Determination of the penicillin concentration in blood and urine Blood was drawn at intervals from the ear veins of th rabbits allowed to stand at room temperature for one hour and then centrifuged The serum and urine peni cillin concentrations were determined with a disc diffusion method according to *Ericsson* (1960)

Experimental procedure Thirtynine white male rabbits weighing between 1 6 and 2 1 kg were used in the experimental series They were observed for at least one week before infection All rabbits were fed with laboratory chow pellets ad libitum

The left ureter was obstructed for 24 hours be fore infection in order to increase the susceptibility to infection and 10^{11} to 2 10^{11} *P vulgaris* in a volume of 25 ml were injected intravesically as reported earlier (*Gnarpe* & *Olding* to be published) After the first day of infection all rabbits were treated with 300000 IU procaine penicillin intramuscularly per kg daily until sacrifice

The rabbits were divided into two groups The animals in group A consisting of 22 rabbits were given unlimited access to tap water The mean daily water intake in this group was 135 ml over the infection period Urine was collected by ca theterization aft r 4 days of treatment at least once more during the infection and at the time of sacrifice 7–10 days later

Group B consisting of 17 animals was treated in the same way as group A for the first four days At this point urine specimens were obtained and then the animals were given unlimited access to 5 5 per cent glucose water for the remainder of the experimental period This procedure has been shown to increase the diuresis to more than twice the normal value (*Andriole* 1968) The mean daily water intake in group B was 140 ml during the first four days and 397 ml during the period of osmotic diuresis The animals were catheter ized once or twice to collect urine during the foll owing 7–10 days after which time they were sacrificed In both groups the kidneys were removed aseptically at post mortem examination as described earlier (*Gnarpe* & *Olding* to be published) and cultures were made for bacteria and spheroplasts

RESULTS

Urine specimens from all rabbits obtained before infection yielded no growth Blood cul tures from 15 animals during the experimen tal period were sterile

Findings after Treatment with Penicillin for Four Days

The results of urine cultures on conventional and on osmotically stabilized media after four days of treatment with penicillin are given in Table 1 Pure cultures of spheroplasts were isolated from 36 of a total of 39 rabbits (92 per cent) From 11 of these 36 rabbits > 10 colony forming units per ml urine were iso lated Mixed cultures of spheroplasts and bacteria were found in specimens from two animals in one rabbit neither bacteria nor spheroplasts were isolated

TABLE 1 *Results of Urine Cultures from 39 Rabbits Infected Intravesically with* P vulgaris *after 4 Days with Penicillin Treatment*

Number of animals 39	
Growth of spheroplasts	36
Growth of spheroplasts and bact ria	2
Growth of bacteria	–
No grow t	1

The concentra of penicillin in blood and urine was 50 J per ml in all rabbits except thre The two cases with growth of 1 d ci s o sph roplasts and

TABLE 2 *The Results of Urine and Kidney Cultures from 22 Rabbits Treated with Penicillin (Group A) and 17 Rabbits Treated with Penicillin and Undergoing Osmotic Diuresis (Group B)*

growth	Group A Number of animals 22		Group B Number of animals 17	
	urine	kidney	urine	kidney
Spheroplasts (only)	19	16	3	7
Spheroplasts and bacteria	–	1	–	–
Bacteria (only)	1	1	–	–
No growth	2	4	14	10

TABLE 3 *Correlation between Urinary pH, Urine Osmolality and the Isolations of Spheroplasts from Urine and Kidney Tissue of 17 Rabbits Undergoing Osmotic Diuresis (Group B)*

Urinary pH	Growth of spheroplasts Urine osmolality					
	< 400 mosm/l			> 400 mosm/l		
	cases	urine	kidney	cases	urine	kidney
< 5 5	3	3	3	–	–	–
5 5–7 0	8	–	4	2	–	–
> 7 0	3	–	–	1	–	–
Total	14	3	7	3	–	–

bacteria had approximately 25 IU per ml in blood and urine The concentration in blood and urine from the rabbit where no microorganisms were isolated was between 50 and 100 IU per ml (MIC for the infecting strain 50 IU/ml)

Findings at Post Mortem Examination

The results of urine and kidney cultures from the rabbits in the two groups A and B are given in Table 2 In group A pure spheroplast cultures were isolated in urine specimens from 19 rabbits (86 per cent) and from one or both kidneys in 16 of these rabbits A mixture of spheroplasts and bacteria was found in kidney tissue from one rabbit The urine culture from this rabbit showed only spheroplasts Bacteria only were isolated from urine and kidney tissue of one rabbit The urine culture from two (9 per cent) and kidney cultures from 4 rabbits (18 per cent) were negative In group B pure cultures of spheroplasts were obtained from the urine and from the kidney tissue of 3 rabbits and from the kidney tissue only from another four animals Urine cultures from 14 animals (82 per cent) and kidney cultures from 10 of these (59 per cent) were negative

Concentration of Penicillin

Group A The concentration of penicillin in blood and urine was between 50 and 100 IU per ml in 16 animals with growth of spheroplasts in urine and kidney tissue and between 100 and 200 IU per ml in the four rabbits *with negative kidney cultures In one rabbit* with growth of spheroplasts in urine and a mixture of bacteria and spheroplasts *in kidney* and in one rabbit with *growth of only* bacteria in urine and kidney *the concentration* was about 25 *IU per ml*

Group B The concentration of penicillin

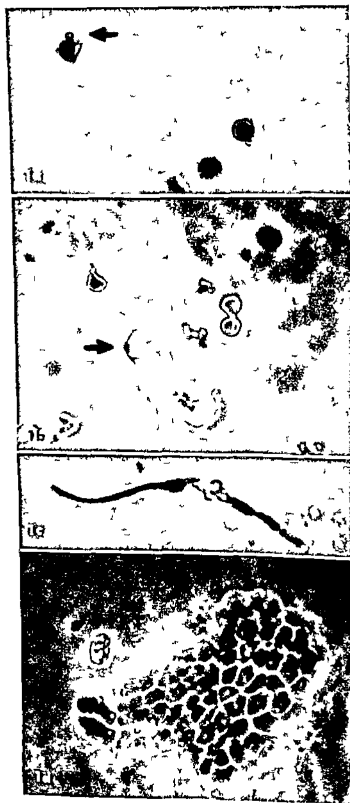


Fig 1 a) Individual spheroplasts on solid CA medium 12 hours after inoculation (× 500) Note budding indicated arrow
b) Single spheroplasts on solid CA medium 16 hours after inoculation Note outgrowth of filament from aberrant spheroplast (× 500)
c) Large aberrant spheroplast in late reversion stage 56 hours after inoculation in fluid CA medium (× 500)
d) Spheroplast colony 3 days after inoculation (× 500)

formed with *E coli* The occurrence of urinary leucocytes was compared with the urinary pH the presence of bacteria and the morphological changes in the urinary tract

MATERIALS AND METHODS

16 white male rabbits weighing between 2 5 and 3 0 kg were used They were fed with laboratory chow pellets and water *ad libitum* Before use they were observed for at least 2 weeks

Bacterial strains The bacteria used in the experiments were *Proteus vulgaris* X 19 obtained from the type culture collection of the National Bacteriological Laboratory Stockholm and an *Escherichia coli 06* strain isolated from the urine of a case of urinary tract infection

Culture The bacteria were cultivated for 18 hours in nutrient broth centrifuged and resuspended in saline to a final concentration of 10^{10} cells per ml by measuring the optical density in a colorimeter (Unicam SP 1300 Colorimeter red filter Ilford No 608 Unicam Instruments Ltd Cambridge England) The inocula contained between 6 10^{10} and 10^{11} cells in a volume of 25 ml

Urinary samples were drawn by catheterization at intervals The urine was cultured on solid media after serial dilutions in saline and incubated at 37 for 24 hours The following solid media were used blood agar (Difco) endo agar (Difco) and for *P vulgaris* desoxycholate citrate agar (Difco) The ability of *P vulgaris* to produce urease was grossly tested by inoculating the bacteria into urea tubes All specimens became positive within 4 hours

To examine the occurrence of bacteria in the kidneys they were weighed and cut in halves One half of one or both kidneys was homogenized by using the X press according to *Edebo* (1968) 1 0 g of kidney homogenate was suspended in 10 ml saline Serial dilutions and cultures were made as above

Leucocyte count The leucocytes in the urine were counted in a Buerker chamber after catheterization Only leucocytes with preserved morphology were included in the count During the transport from the operating room to the laboratory the urine samples were kept in ice water The time interval between collection and examination never exceeded 25 min

Measurement of the pH The hydrogen ion concentration in the urine was measured by a combined glass-calomel electrode (Type GK 2071 C) connected to a pH meter (Titrator Type TTT 1 Radiometer Copenhagen

Histological examination Histological examination of the urine bladder the renal pelves and both kidneys were performed in 10 of 12 rabbits infected with *P vulgaris* and in 3 of 4 rabbits infected with *E coli* The organs were fixed in 10 per cent formaldehyde and sections were made from various parts of them van Gieson and haematoxylin – eosin stains were used for histological studies and Giemsa and Gram stains for visualization of bacteria in the sections

Experimental procedures The rabbits were anaesthesized intravenously with Nembutal (20–40 mg per kg) and additional local anaesthesia in skin and muscle layers (Xylocain 0 1 per cent 2 ml) In order to increase the susceptibility to infection the left kidney was exposed through an incision in the loin and the ureter was temporarily obstructed by a silk thread which was passed around the ureter and then through the muscle and skin layers The suture was knotted around a piece of rubber tube as modified from *Gorrill (1956)* and *Prat et al (1959)* The muscle and skin layers were closed with interrupted catgut and silk sutures respectively

The suture obstructing the ureter was removed after 24 hours to permit a free flow of urine A sterile disposable catheter (Mediplast No FG 12) was inserted into the bladder under light Nembutal anaesthesia and the urine in the bladder was collected aseptically for culture measurement of the pH and for leucocyte count The bacteria *P vulgaris* or *E coli* were then injected into the bladder through the catheter in a volume of 25 ml The catheter was removed and the bladder was gently squeezed 20 to 25 times through the abdominal wall in order to obtain ureteral reflux while the external urethral meatus was compressed between the finger tips as described by *Prat* (1967)

In order to study the progress of the infection the rabbits were catheterized under light Nembutal anaesthesia as described above the urine was drawn into sterile flasks immediately cooled in ice water and then divided in different portions for pH measurement leucocyte count and quantitative culture From each rabbit except one between two and six specimens were collected The animals were sacrificed at intervals from 2 to 11 days after infection They were killed by an overdose Nembutal intravenously The kidneys the ureters and the bladder were aseptically removed through a midline incision in the abdominal wall

RESULTS

Findings before Infection

All urine specimens taken immediately before the injection of bacteria into the bladder were sterile save one which showed growth of [illegible] *E coli* per ml All urine sam[illegible] collected before the infection con

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THE INFLAMMATORY REACTION AND URINARY LEUCOCYTES IN ASCENDING URINARY-TRACT INFECTIONS

An Experimental Study on Rabbits Infected with Proteus vulgaris X 19 *and* Escherichia coli 06

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Ascending urinary tract infections with Proteus were induced in rabbits by intravesical inoculation of bacteria. The urinary findings of leucocytes, bacteria, alterations in the urinary pH and the morphological lesions in the urinary tract were studied. In spite of heavy growth of bacteria in urine and in the kidneys and inflammatory lesions in the urinary tract there were remarkably low concentrations of leucocytes in the urine at pH values above 7.75. This was the case in most of the rabbits infected with *P. vulgaris*. When the urinary pH was below 7.75 there was a considerable concentration of leucocytes. This was observed in some of the rabbits infected with *P. vulgaris* and in most of those infected with *E. coli*. The demonstration of leucocytes in urinary sediments as a criterium of urinary tract infection must be reconsidered, especially in infections due to proteus bacteria, as there is evidence that they cause leucocyte disintegration *in vivo*.

The study of urinary sediments is a valuable aid in the diagnosis of suspected urinary tract infections. Infections of the urinary tract are usually accompanied by a leucocyte excretion which leads to pyuria (*Little* 1965, *Fairley* & *Ravenscroft* 1957).

However, the findings of leucocytes in urine sediments is [illegible] by several factors: 1) A considerable delay between collection and examination of the urine specimens, especially if the urine is kept at room temperature (*McIntyre* & *Mou* 1965, *Triger* & *Smith* 1966), 2) hypotonic urine (*McIntyre* & *Mou* 1965) and 3) alkaline urine (*Little* 1965, *Gnarpe* & *Edebo* 1965, *McIntyre* & *Mou* 1965). These factors, single or combined, might cause disintegration of exudated leucocytes.

In infections of the urinary tract with bacteria belonging to the genus Proteus the urinary pH often exceeds 7.0 due to the splitting of urea by bacterial urease, and the sediment findings of leucocytes are often sparse and irregular (*Sandford* 1956, *Edebo* & *Laurell* 1958). These observations initiated experiments with ascending urinary tract infections with proteus bacteria in rabbits to study the acc[illegible]lation, exudation and stability of le[illegible] the infected areas. For comparison [illegible] nts were per

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The authors wish to thank Mr. C. Ek[illegible], Ph. D., for valuable help with the statistical calculations.

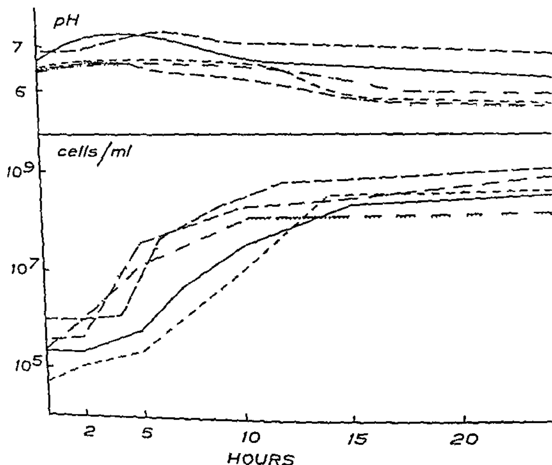


Fig 2 Influence of bacterial growth on the hydrogen ion concentration in urine with 100 mg % glucose added

Analogous experiments were performed with *P vulgaris Ps aeruginosa Alc faecalis E coli Staph albus* and *Str faecalis* inoculated into urine to which glucose had been added to a concentration of 1 mg/ml (Fig 2) After an initial increase *P vulgaris* and *Ps aeruginosa* caused a decrease in the pH to 6 25 and 6 75 respectively, after 24 hours. The other organisms tested lowered the pH from 6 50 to below 6 0

Experiments were also performed with *P vulgaris Ps aeruginosa Alc faecalis E coli Staph albus* and *Str faecalis* inoculated into the synthetic medium described under methods The results did not differ significantly from the results of bacterial growth in urine without glucose

When *P vulgaris* and *E coli* were inoculated together in urine at an initial ratio between the number of *E coli/P vulgaris* of about 10/1 there was no increase in the urinary pH (Fig 3a) When the *E coli/P vulgaris* ratio approximately 1/1 or 1/10 (Figs 3b and 3c respectively) there was a rapid increase in the urinary pH to levels above 9 0

DISCUSSION

The pH values of normal urine has wide limits i e pH 4 8-8 0 As a result of the renal excretion of acid ash products it is usually lightly acid (5 5 7 0) The experiments described in his communication ... shown that the pH ... influenced by

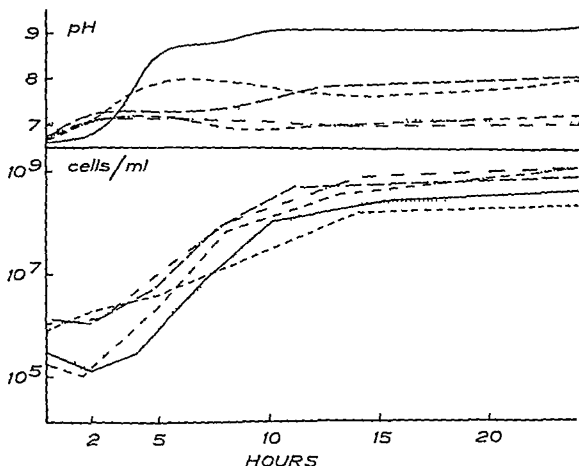


Fig 1 Influence of bacterial growth on the hydrogen ion concentration in urine

——— *P vulgaris* — — *E coli*
— —— *Ps aeruginosa* — — — *Staph albus*
— — — — *Alc faecalis* *Str faecalis*

The pH was measured by a combined glass alomel electrode (type GK 2021 C) immersed in the culture medium connected to a pH meter (Titrator Type TTT 1 Radiometer Copenhagen) The pH was continuously registered by connecti the pH meter to a paper recorder (Ela vi 3) The temperature was kept at 37 ie aid of a contact thermometer connected heating tape wound around the vessel via an tronic relay The medium was stirred with a lon coated magnet during cultivation

Measurem nt of bacterial concentration The ptical density of the culture was measured in a pectrophotometer (Beckman DB) at 660 mμ by pumping the growth medium through an o erflow optical cell and continuously recording the optical den ity by connecting the spectrophotome r to a paper recorder (Beckman 93507 recorder

At certain intervals samples were taken for viable counts Viable counts were ma le after serial dilutions in sa in by plating 0 1 ml amoun s on solid medi f lowing media were use for *P vulgaris* desoxycholate citrate agar (Difco) for *E coli* endoagar (Difco) for all the others nutrient agar (Difco)

RESULTS

The growth of pure cultures of *P vulgaris Ps aeruginosa Alc faecalis E coli Staph albus* and *Str faecalis* in urine gave the results shown in Fig 1 Within 5 hours Proteus rai ed the pH to 8.5 before any growth was noted After 10 hours when the growth was slowing down the pH was more than 9 0 As regards the other organisms tested *Ps aeruginosa* and *Alc faecalis* caused a rise of the pH to around 8 0 *Str faecalis E coli* and *Staph albus* caused an increase of the pH to 7 15 7 05 and 7 25 respectively

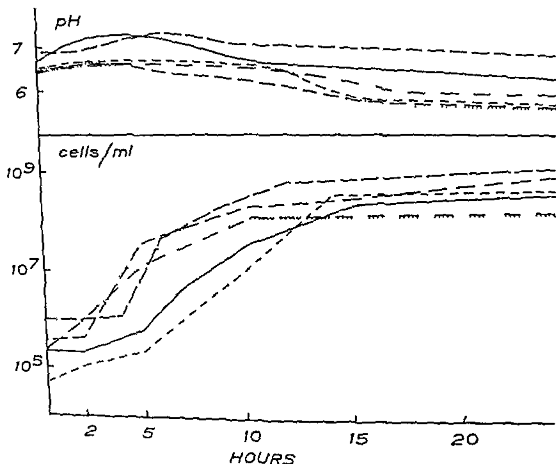


Fig 2 Influence of bacterial growth on the hydrogen ion concentration in urine with 100 mg % glucose added

Analogous experiments were performed with *P vulgaris Ps aeruginosa Alc faecalis E coli Staph albus* and *Str faecalis* inoculated into urine to which glucose had been added to a concentration of 1 mg/ml (Fig 2) After an initial increase *P vulgaris* and *Ps aeruginosa* caused a decrease in the pH to 6 95 and 6 75 respectively after 24 hours The other organisms tested lowered the pH from 6 50 to below 6 0

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When *P vulgaris* and *E coli* were inoculated together in urine at an initial ratio between the number of *E coli/P vulgaris* of about 10/1 there was no increase in the urinary pH (Fig 3a) When the *E coli/P vulgaris* ratio approximately 1/1 or 1/10 (Figs 3b and 3c respectively) there was a rapid increase in the urinary pH to levels above 9 0

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THE INFLAMMATORY REACTION AND URINARY LEUCOCYTES IN ASCENDING URINARY-TRACT INFECTIONS

An Experimental Study on Rabbits Infected with Proteus vulgaris X 19 *and* Escherichia coli *06*

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Ascending urinary tract infections with Proteus were induced in rabbits by intravesical inoculation of bacteria The urinary findings of leucocytes bacteria alterations in the urinary pH and the morphological lesions in the urinary tract were studied In spite of heavy growth of bacteria in urine and in the kidneys and inflammatory lesions in the urinary tract there were remarkably low concentrations of leucocytes in the urine at pH values above 7 75 This was the case in most of the rabbits infected with *P vulgaris* When the urinary pH was below 7 75 there was a considerable concentration of leucocytes This was observed in some of the rabbits infected with *P vulgaris* and in most of those infected with *E coli* The demonstration of leucocytes in urinary sediments as a criterium of urinary tract infection must be reconsidered especially in infections due to proteus bacteria as there is evidence that they cause leucocyte disintegration *in vivo*

The study of urinary sediments is a valuable aid in the diagnosis of suspected urinary tract infections Infections of the urinary tract are usually accompanied by a leucocyte excretion which leads to pyuria (*Little* 1965 *Fairley* & *Barraclough* 1967)

However, the finding of leucocytes in urine sediments is jeopardized by several factors 1) A considerable delay between collection and examination of the urine specimens, especially if the urine is kept at room temperature (*McIntyre* & *Mou* 1965 *Triger* & *Smith* 1966), 2) hypotonic urine (*McIntyre* & *Mou* 1965) and 3) alkaline urine (*Little* 1965 *Gnarpe* & *Edebo* 1965 *McIntyre* & *Mou* 1965) These factors, single or combined might cause disintegration of exudated leucocytes

In infections of the urinary tract with bacteria belonging to the genus Proteus the urinary pH often exceeds 7 0 due to the splitting of urea by bacterial urease and the sediment findings of leucocytes are often sparse and irregular (*Sandford* 1956, *Edebo* & *Laurell* 1958) These observations initiated experiments with ascending urinary tract infections with proteus bacteria in rabbits to study the accumulation exudation and stability of leucocytes in the infected areas For comparison analogous experiments were per

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An Experimental Study on Rabbits Infected with Proteus vulgaris X 19 *and* Escherichia coli *O6*

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Fig 3 Papillitis of the kidney Clumps of leucocytes in the lumina of the collecting tubuli near the orifices of the tubuli in a renal papilla (Case 7 Table 1) H & E × 120



Fig 4 Necrotizing papillitis of the kidney Area of necrosis in a renal papilla denoted with arrows (Case 15 Table 2) H & E × 30

by *E coli* (cases 15 and 16) 2) *Papillitis* comprising clumps of leucocytes beneath the epithelium covering the papillae or in the orifices of the collecting tubuli (Fig 3) The papillitis was usually combined with necrosis of the tubuli and the interstitial tissue of the papillae (necrotizing papillitis) (Fig 4) Four animals in the proteus series showed this change (case No 3 6 7 and 11) These changes were also found in two out of the three rabbits infected with *E coli* whose kidneys were examined morphologically (cases 15 and 16) In the cases of additional pyelonephritis the term papillitis always meant necrotizing papillitis (cases 3 15 and 16) 3) *Pyelonephritis* with streaks of foci of leucocytes in the tubuli and in the interstitial tissue of the renal medulla and cortex (Fig 5a b) There were many eosinophilic leucocytes in the exudate Two cases in the proteus infected series (cases 2 and 3) and two in the *E coli* infected series (cases 15 and 16) showed these changes 4) Large *infarctions* bordered by heavy inflammation and taking in large areas of both cortex and medulla of the kidney These changes were found in two cases No 9 and 13 It is feasible that these changes were produced by traumatic vascular lesions at the operation

The different types of morphological changes were often combined

tained no leucocytes except two in which a few leucocytes were seen. In these cases there were also erythrocytes in the urine indicating a slight damage during the catheterization. Repeated blood cultures from 12 infected animals were sterile.

In the proteus infected group the mean urinary pH before infection was 7.59 and the group infected with *E. coli* 7.68.

Bacterial Growth

Urine cultures yielded growth of 10^5 to 2×10^5 bacteria per ml in the rabbits infected with *P. vulgaris*. In the rabbits infected with *E. coli* the bacterial counts were between 10^5 and 2×10^5 bacteria per ml urine.

Relation between Leucocytes Concentration and Urinary pH

In 25 of 40 urine samples (63 per cent) from the proteus infected group pH values above 7.75 were observed (Fig. 1*). In all of these 25 samples fewer than 100 leucocytes per mm^3 were found and in 22 (55 per cent) fewer than 50 per mm^3. The remaining 15 samples (38 per cent) from the proteus in-

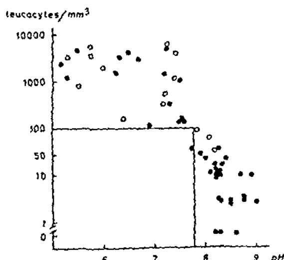


Fig. 1. Concentration of urinary leucocytes in relation to urinary pH values in rabbits infected with *P. vulgaris* (●) and rabbits infected with *E. coli* (○) on different occasions.



Fig. 2. Cystitis (of moderate degree). Foci of leucocytes both in the surface epithelium and around small vessels in the wall of the urinary bladder (arrows). (Case 3, Table 1). H & E. × 120.

fected animals had a pH below 7.60; in 10 of these samples there were 1000 leucocytes per mm^3 or more.

In 3 of 14 observations in the *E. coli* infected rabbits (22 per cent) there were pH values above 7.70. In these observations fewer than 100 leucocytes per mm^3 were found. The remaining 11 observations had pH values below 7.70 and more than 100 leucocytes per mm^3.

Morphological Lesions

Histological examinations revealed four main types of morphological changes. 1) *Cystitis* which was predominantly focal and of a slight to moderate degree. The epithelium of the bladder was well preserved but infiltrated by leucocytes (Fig. 2). This change was found in five rabbits infected by *P. vulgaris* (case No. 2, 3, 4, 5 and 12) and in two infected

* All car the authors for details.



Fig 3 Papillitis of the kidney Clumps of leucocytes in the lumina of the collecting tubuli near the orifices of the tubuli in a renal papilla (Case 7 Table 1) H & E × 170



Fig 4 Necrotizing papillitis of the kidney Area of necrosis in a renal papilla denoted with arrows (Case 15 Table 2) H & E × 30

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Urine Examinations

The pH val r ~ sacrif the '

tube which was kept in ice water for different periods of time varying from 0 to 120 min The bacteria were collected by centrifugation at 6000 × g/10 min/0 C washed once in KRG and resuspended in KRG containing 5 per cent serum (10^9 bacteria per ml)

As controls ³²P labelled *E coli* were treated in the same way except that KRG was used instead of the strangulation fluid filtrate

Strangulation Fluid Filtrate Adsorbed with Unlabelled E coli

Unlabelled *E coli* were suspended into undiluted filtrate (5×10^{10} per ml) The suspension was kept at 0 C for 60 min The bacteria were removed by centrifugation at 6000 × g/10 min/0 C and the process repeated with another portion of bacteria Following the second centrifugation the supernatant was passed through a Millipore filter with pore size 0 22 μ

Preincubation

A mixture of 5 per cent serum and 10 per cent of the filtrate in KRG was preincubated at 37 C for 60 min (8) Control medium containing 5 per cent serum in KRG was preincubated in the same way Subsequently ³²P labelled *E coli* were suspended into each medium (10^9 per ml) in which the release of label was determined

Determination of Release of Label into the Medium

The technique used has been described elsewhere (8 9 11)

Statistical Methods

The two samples ranks test of Wilcoxon White was used (3 9)

RESULTS

Labelled bacteria pretreated in late strangulation fluid filtrate were subsequently incubated in a medium containing serum The release of label from bacteria pretreated in this way was larger than the release from control bacteria pretreated in KRG only (Fig 1) The release of label was dependent upon the time of pretreatment in the filtrate At 0 min the release of ³²P was not different from the controls ($p > 0.10$) Following exposure to the filtrate for 30 min the bacteria lost 20 per cent more radioactivity than the controls ($0.05 > p > 0.01$) Pretreatment in the filtrate for 120 min resulted in a release of label which was 38 per cent larger than the controls ($p < 0.01$)

Late strangulation fluid filtrate and

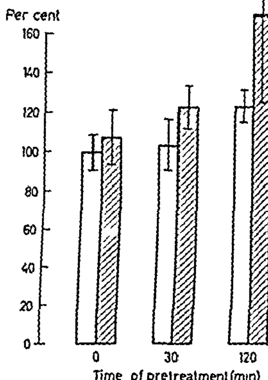


Fig 1 Release of ³²P from labelled *E coli* pretreated in late strangulation fluid filtrate for various periods of time at 0 C ³²P labelled *E coli* were pretreated in the filtrate as described in Materials and Methods The bacteria were subsequently suspended in KRG with 5 per cent serum (10^9 per ml) Aliquots (2 5 ml) of the suspension were incubated at 37 C for 15 min As controls ³²P labelled *E coli* exposed to KRG instead of the filtrate were treated in the same way The release of label into the medium was determined Each column represents the mean of eight observations (two experiments) ± standard deviation 100 per cent = the release of label from *E coli* suspended in KRG and immediately centrifuged prior to being tested in KRG with 5 per cent serum

sorbed with large numbers of unlabelled *E coli* were preincubated with serum and the capacity of this mixture of releasing ³²P from labelled bacteria was determined (Fig 2) In this medium the release of label was approximately 50 per cent smaller than the release obtained in a similarly preincubated medium

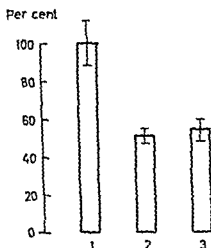


Fig 2 The inhibitory effect of adsorbed and untreated late strangulation fluid filtrate on the ^{32}P releasing activity of serum Late filtrate was adsorbed with *E coli* as described in Materials and Methods and preincubated with serum ^{32}P labelled *E coli* were suspended into each medium to be tested (10^9 per ml) The media consisted of 1) 5 per cent serum in KRG (control) 2) 10 per cent adsorbed filtrate and 5 per cent serum in KRG and 3) 10 per cent untreated filtrate and 5 per cent serum in KRG Aliquots (2 5 ml) of each suspension were incubated at 37 C for 15 min whereafter the release of label into the medium was determined Each column illustrates the mean of ten individual observations (two experiments) ± standard deviation expressed as per cent of the release observed in medium 1

in which the filtrate was absent The release of label was not significantly different whether the medium contained adsorbed filtrate or untreated filtrate ($p > 0\ 10$)

DISCUSSION

Fresh serum has a bactericidal effect on *E coli in vitro* (4) The bactericidal activity of serum can be estimated indirectly by measuring the release of label into the medium from ^{32}P labelled *E coli* (7 11) The amount of label released under such conditions varies inversely with the number of remaining viable bacteria The reaction is believed to result from complement activation during which lesions are produced in the bacterial wall Lysozyme enhances the release of label initiated by complement (12)

Late strangulation fluid might contain substances which stimulate the bactericidal activity of serum as well as substances which inhibit this reaction The results of the current investigation show that prolonged exposure of ^{32}P labelled *E coli* to late strangulation fluid filtrate at 0° C alters the sensitivity of the bacteria to the ^{32}P releasing activity of serum The findings indicate that the filtrate contains substances which stimulate the bactericidal activity of serum It may be recalled that early strangulation fluid filtrate also seemed to contain factors which were capable of enhancing the bactericidal capacity of serum yet being unable to initiate the reaction by itself (9)

In a previous report (8) it was demonstrated that late strangulation fluid filtrate contained substances which partly inactivated the bactericidal capacity of serum These substances might act either by interfering with serum components in the fluid phase, or by a more direct effect upon the bacteria Coating of bacteria with such substances might protect the bacteria from the bactericidal activity of serum If so one would expect that the inhibitory substances could be removed from the filtrate by adsorption with large numbers of bacteria The results clearly demonstrated however that the inhibitory effect of the filtrate on serum components was unaltered by such adsorption This fact indicates that the inhibitory substances affect the serum components directly and not via the bacteria

Other experiments have revealed that strangulation fluid collected from germfree rats also contains substances which inhibit the bactericidal activity of conventional rat serum (10) The inhibitory substances are therefore probably released from host tissues rather than from bacteria Although the results do not allow conclusions to be made concerning the nature of these inhibitory substances they appear to provide a basis for further studies of these pathophysiologically important problems

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germfree rats is not lethal when injected into the peritoneal cavity of test mice (2, 9)

Rat canine and human strangulation fluids lose the lethal property when bacteria are removed by filtration (3 4, 23) The sterile, nonlethal filtrate has the property of enhancing the virulence of *E coli* (1, 7) and other bacteria (6) Smaller numbers of *E coli* are required to kill assay mice upon intraperitoneal injection when the bacteria are suspended into filter sterilized strangulation fluid, than when other suspending media are used, such as normal saline or rat plasma (1) The virulence enhancing property is probably not due to bacterial products since *Amundsen* (1) was able to demonstrate that strangulation fluid from germfree rats also enhanced the virulence of *E coli*

The effects of strangulation fluid filtrates from conventional rats on host defence reactions have been studied previously (20, 21) Sterile filtrates of such fluid show less opsonic and bactericidal activities on *E coli* than does normal rat serum (21) The filtrate of fluid obtained later than 48 hours after operation also appears to interfere with the opsonic and bactericidal capacities of normal rat serum (20) The presence in the filtrate of inactivators of either antibody or complement components seems likely

Substances capable of inactivating serum components might be released from bacteria or from the tissues of the diseased animal Germfree rats seemed to be suitable for study g the origin of these substances

In the present study strangulation fluid collected from germfree rats was used for studying aspects of host defence The following parameters were investigated 1) The virulence enhancing effect of such fluid on *E coli* towards mice 2) Phagocytosis of ³P labelled *E coli* by rat polymorphonuclear leucocytes in the presence of strangulation fluid from germfree rats 3) The bactericidal activity of media containing such fluid on *E coli* The bactericidal capacity was measured indirectly by determining the release of radioactivity from ³P labelled *E coli* into the medium It has repeatedly been demonstrated that the amount of label released during such conditions varies inversely with the number of remaining viable bacteria (19 22)

MATERIALS AND METHODS

Animals

Germfree (GF) rats of the CDF strain (Charles River Breeding Lab Wilmington Mass) were reared as described by *Midtvedt & Trippestad* (15)

Conventional rats used as serum and cell donors were of local strain kept under standard laboratory conditions

Strangulation Fluid

GF rats were anaesthetized with sodium pentobarbital (Nembutal® Veterinary Abbott Laboratories Kent England) and operated upon inside the isolators 16 rats of both sexes were used between 80 and 120 days of age The operative technique was similar to the one used in conventional rats (3) except that strangulation fluid was collected at room temperature and removed from the isolator every 24 hours

The fluid portions were pooled in three fractions The early fluid consisted of portions collected during the first 24 hour period The late fluid was pooled from portions produced later than 48 hours after the operation The fluid collected between 24 and 48 hours was not used in the current experiments

The pooled GF fluid was centrifuged at 100 000 × g/60 min/0 C and the supernatant filtered through a Millipore® filter with pore size 0 22 µ and stored at —70 according to methods previously employed in preparing filtrates of strangulation fluid from conventional rats (21)

Serum

Conventional serum was obtained from conventional rats (22) and GF serum from GF rats (15) The sera were pooled from several rats

Polymorphonuclear Leucocytes (PMN)

PMN were harvested from the peritoneal cavity of conventional rats following stimulation with 1? per cent sodium caseinate (??)

Bacteria

The strain of *E coli* was the same as the one used in other studies (14 15 ?0 21 ??) Unlabelled bacteria were cultured in a casein medium (5) Labelled bacteria were cultured in the same medium to which was added 1 mCi ³P labelled orthophosphate as described in another report (22)

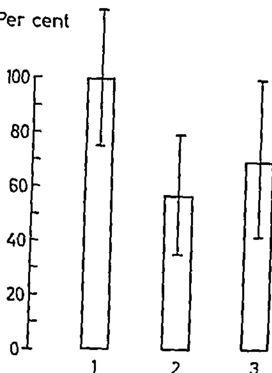


Fig 1 Phagoc tosis of ³ P labelled *E co'i by* PMN incubated in media of different composition Aliquots (2 5 ml) of a suspension of ³ P labelled *E coli* (10⁹ per ml) in the medium to tested were incubated for 15 min at 37 C in tissue culture tubes containing monolayers of PMN The media consisted of 1) KRG with 10 per cent GF serum *(control)* 2) KRG with *10* per cent early GF *strangulation fluid filtrate* and *3)* KRG with 10 per cent late GF strangulation fluid filtrate Each column represents the mean of ten individual observations in two experimental series expressed as per cent of the controls The bars indicate ± one standard deviation

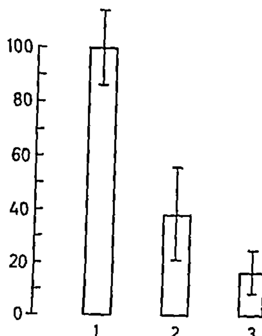


Fig ² Phagocytosis of ³ I labelled *E coli* by PMN incubated in media *containing GF strangulation fluid filtrates* and *conventional* serum preincubated *at 37* C The test media and controls were preincubated at 37 C for 60 min prior to the addition of ³ P labelled *E coli* Aliquots (2 5 ml) of a suspension of labelled *F coli* (10³ per ml) in the medium to be tested were subsequently incubated at 37 C for 15 min in tissue culture tubes containing monolayers of PMN The media consisted of 1) KRG with 5 per cent conventional serum (control) 2) KRG with 10 per cent early GF strangulation fluid filtrate and 5 per cent conventional serum and 3) KRG with 10 per cent late GF strangulation fluid filtrate and 5 per cent conventional serum Each column represents the mean of ten individual observations in two experimental series expressed as per cent of the controls The bars indicate ± one standard deviation

ie medium from ³ P labelled *E coli* following incubation in 10 per cent of either GF rangulation fluid filtrate, or in the same mount of GF serum The release produced i the presence of either filtrate was about ne third of that found in GF serum

The influence of the GF filtrates on the oility of conventional serum to release la elled substances from the bacteria into the medium will be seen from Fig 4 A mixture of early GF filtrate and conventional serum which had been preincubated as described produc ι rι se of ıbel during subsequent incubation with ³ P labelled *L coli* which was approximately 50 per cent of that of the control When a mixture of late GF filtrate and conventional serum was treated in the same way the subsequent release was only 33 per cent of the control The difference between the property of the early and that of the late filtrate of interfering with the ³ P releasing power of serum was highly significant ($p < 0.01$)

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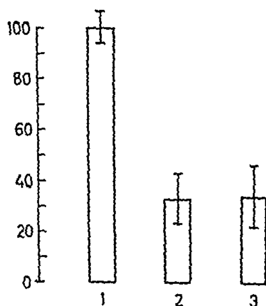


Fig 3 Release of ^{32}P from labelled *E coli* incubated in GF serum or in GF strangulation fluid filtrates Aliquots (2 5 ml) of a suspension of ^{32}P labelled *E coli* (10^9 per ml) in the medium to be tested were incubated for 15 min at 37 C The media consisted of 1) KRG with 10 per cent GF serum (control) 2) KRG with 10 per cent early GF strangulation fluid filtrate and 3) KRG containing 10 per cent late GF strangulation fluid filtrate Each column represents the mean of ten individual observations in two experimental series expressed as per cent of the controls The bars indicate ± one standard deviation

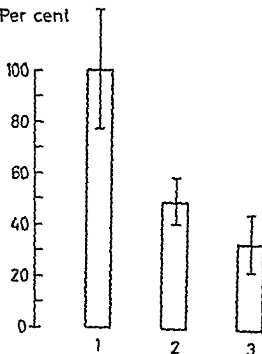


Fig 4 Release of ^{32}P from labelled *E coli* during incubation in media containing GF strangulation fluid filtrates and conventional serum preincubated at 37 C The media were preincubated at 37 C for 60 min prior to the addition of ^{32}P labelled *E coli* Aliquots (2,5 ml) of a suspension of labelled *E coli* (10^9 per ml) in the medium to be tested were incubated at 37 C for 15 min The media consisted of 1) KRG with 5 per cent conventional serum 2) 10 per cent early GF strangulation fluid filtrate and 5 per cent conventional serum in KRG and 3) KRG with 10 per cent late GF strangulation fluid filtrate and 5 per cent conventional serum Each column represents the mean of ten individual observations in two experimental series expressed as per cent of the controls The bars indicate ± one standard deviation

DISCUSSION

The virulence enhancing activity of strangulation fluid filtrates from conventional rats on *E coli* is present only in portions of strangulation fluid produced during the later stages of the ailment and cannot be demonstrated in portions collected during the first 24 hour period (3 14) The cause of the enhanced virulence is as yet unknown A direct effect on the bacteria or interference with host defence reactions are both possible explanations *E coli* multiply rapidly in filtrates of strangulation fluid from conventional rats The growth rate alone cannot however explain the virulence enhancing effect (14)

Amundsen (1) found that strangulation fluid collected from GF rats enhanced the virulence of *E coli* He concluded that the enhanced virulence was not caused by bacterial products In his experiments however the GF strangulation fluid was not fractionated into early and late portions

The results of the present study show that even the early GF strangulation fluid enhances the virulence of E coli This finding might indicate that the virulence-enhancing factor(s) appears ea[illegible]

fluid from GF rats than it does in such fluid from conventional rats

Previous experiments have shown that in strangulation fluid filtrates from conventional rats phagocytosis of *E coli* by PMN is rather small This is probably a result of insufficient opsonization of the bacteria, since the filtrates do not seem to exert a toxic effect on the PMN (21) Both 'early' and 'late' GF strangulation fluids provide less stimulation of phagocytosis than that obtained with serum from GF rats This indicates the lack of sufficient opsonic activity in the filtrates

The small release of label from the bacteria into media containing strangulation fluid filtrates from GF rats also indicates that humoral serum components might be absent in the filtrates Serum components of the antibody complement system are believed to represent necessary factors in the opsonic and bactericidal reactions of serum One or more of these components may not be sequestered in strangulation fluid, or inactivation of such components might take place due to interaction with other substances in the filtrates

In an earlier report (20) it was shown that 'late' strangulation fluid filtrate from conventional rats interfered with the capacity of rat serum of stimulating phagocytosis and extracellular disintegration of *E coli*, while the 'early' filtrate did not From the results of the present investigation it appears that the opsonic and bactericidal activity of conventional rat serum is substantially lowered by the 'early' as well as the 'late' GF strangulation fluid

The strong inhibitory effect of 'early' GF fluid on the opsonic and bactericidal activities of serum indicates that substances are released from host tissues even in the early phase of intestinal strangulation obstruction These substances seem to interfere with antibacterial defence mechanisms Since the 'early' strangulation fluid from conventional rats does not have similar effects it might be assumed that the inhibitory substances are partly destroyed in conventional rats Further studies are required to elucidate this problem

It is reasonable to assume that decreased resistance towards bacterial infection develops during intestinal strangulation obstruction The substances which are responsible for the decreased resistance are probably released from host tissues rather than from bacteria

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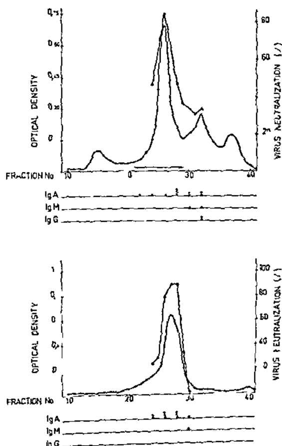


Fig 5 Bio Gel A 15 elution pattern (a) of 2 ml reduced alkylated humna whey sample The column 1 2 cm × 160 cm was equilibrated with 0 05 M phosphate buffer pH 7 2 containing 0 2 M NaCl The double arrow in the figure indicates fractions which were pooled and rechromatographed on the same column (b) The re ults of immunodiffusion after concentrating the fractions about 5 times are shown below the curves Filled circles denote absorbancy at 280 mμ triangles poliovirus neutralizing activity

Electron Microscopy of High Polymer IgA

High polymer IgA preparations tended to form small aggregates when dried on to the carbon coated grid with the negative contrast stain (Fig 10) In the peripheral part of such looser aggregates free laying particles apparently representing high polymer IgA molecules were present in high concentration Only after extensive examination of the preparations was it possible to recognize particles with recurr uniform molecular configuration Three, or more commonly four, filamentous arms protruded from the central part of such molecules and the total span of the molecules was approximately 100 Å (Fig 11) The dimensions of the flexible filamentous extensions were 50 to 55 Å × 15 to 20 Å An increased width (25 Å) was often indicated at the peripheral ends of these extensions

DISCUSSION

Several different procedures for the purification of colostral IgA have been described in recent years Most investigators have started with the removal of fat and casein by high speed centrifugation Casein has occasionally been precipitated by lowering the pH to 4 5-4 6 The supernatants have subsequently been subjected to purification procedures of vary

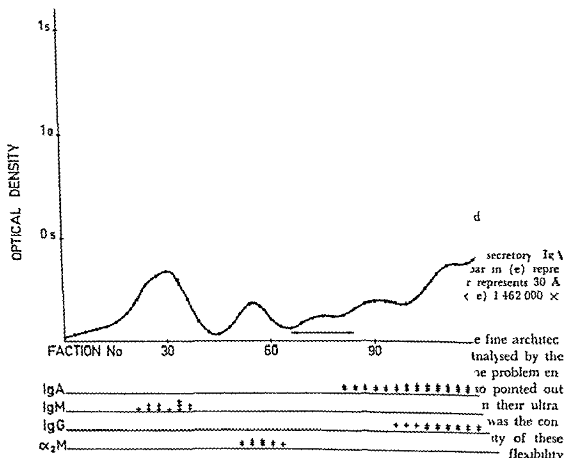


Fig 6 Elution pattern (major part) of ?0 ml untreated human serum on two Bio Gel A 15 tinctly a coupled in series The columns ? 5 cm × 400 cm and 2 5 cm × 500 cm were equilibrated whole same phosphate buffer as used in other gel filtration runs The double arrow indicates the fract of which were pooled concentrated and submitted to further purification The results of immunodiffusio after about 10 times concentration of fraction are shown below the elution patterns

investigators (8 24 9 36 32) the IgA being both of normal and myeloma type. In con trast complex formation between s IgA and albumin has not been described *Heremans* (25) suggested that albumin only binds to aggregated serum IgA and that the ageing of serum is a contributing factor In the present investigation where purification of IgA was initiated within a few hours after its collec tion refiltration of purified s IgA or micro scopic examination of such material provided no evidence of s IgA aggregation The s IgA albumin complexes could be dissociated by mild reduction in accordance with results from studies (7 8 24) of serum IgA and IgM albumin complexes The possible parti cipation of noncovalent bonds dependent on intact disulphide bonds in this complex for mation was not ruled out either in our study or in the studies mentioned above However *Mannik* (32) has recently shown conclusively that IgA globulins complex with serum albu min through formation of intermolecular disulphide bonds

Secretory IgA preparations derived from a number of colostral samples collected from healthy young Swedish women demonstrated antibody activity against poliovirus type I in low titres These women had been properly immunized parenterally with killed polio

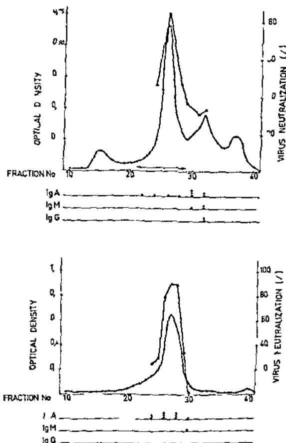


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DISCUSSION

Several different procedures for the purification of colostral IgA have been described in recent years Most investigators have started with the removal of fat and casein by high speed centrifugation Casein has occasionally been precipitated by lowering the pH to 4 5–4 6 The supernatants have subsequently been subjected to purification procedures of varying complexity *Sell* (38) relied upon a single gel filtration step on Sephadex G 200 while other investigators (43 11 22 6) have employed from one up to four further purification steps

Many of the techniques described have the disadvantage of being either rather time consuming or would seem to yield IgA preparations of relatively low purity The technique used in the present study is easy to perform since it involves only two (or three) steps centrifugation, (reduction alkylation) and gel filtration on tall Bio Gel A 15m columns. The s IgA preparations obtained satisfied even the rather high requirements for purity which must be laid on a material to be used for electron microscopic analyses A slight contamination with IgM is often difficult to avoid in colostral IgA preparations No IgM was however detected by double diffusion analyses or the Mancini technique in the s IgA samples (from the s-IgA peak on the A 15 m columns) employed for electron microscopy in the present study But a minor contamination of albumin apparently complexed with IgA, was noted in some s IgA batches The occurrence of serum IgA albumin complexes has been reported by other

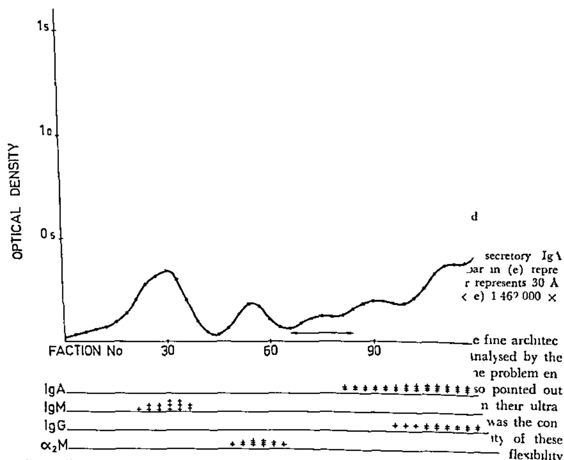


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Secretory IgA preparations derived from a number of colostral samples collected from healthy young Swedish women demonstrated antibody activity against poliovirus type *1* in low titres These women had been properly immunized parenterally with killed polio

duce variations in the number of non viable cells in the thymolymphatic system, and furthermore whether a change in degree of decay could be correlated to the immuno suppressive effect of ALS The aim of the present study is an investigation of the short time effects of a single dosis of antithymocytic serum (ATS) given to mice

MATERIALS AND METHODS

Preparations and Properties of Serum

The ATS was prepared with thymocytes according to descriptions by *Levey & Medawar* (16) Only serum obtained 3 weeks after the start of immunization was used The serum was kept under sterile conditions heated to 56 C for ½ hour in waterbath and stored at —20 C Normal rabbit serum (NS) was treated in the same way *In vitro* cytotoxity was estimated as described by *Abaza & Woodruff* (1) Final dilution of the last tube showing more than 15 per cent stained cells was taken as the cytotoxic titre this was found to be 1/256 Control tubes showed less than 0 per cent stained cells

The leuco-agglutination test was also performed as described by *Abaza & Woodruff* (1) and percentage of agglutination was calculated as 100 × (total number of cells free cells)/total cells Titre was expressed as the reciprocal of the highest dilution giving more than 20 per cent agglutination and was found to be 1/512 All control values were less than 10 per cent

The immunosuppressive activity of the serum was tested by skin grafting Balb/C mice received C_3H skin grafts and were injected with 0 5 ml of ATS 2 and 3 days after transplantation The median graft survival time turned out to be 31 days compared to 12 days in the case of non injected mice

General

Balb/C mice weighing 25–34 gms in equal numbers of males and females were examined The animals were kept under standard conditions They were separated in groups according to each experiment One group of 10 animals remained uninjected and was examined as controls during the entire experimental period The test material was divided into two major groups each having 20 mice The first major group received 0 5 ml ATS in the subcutis of the infrascapular region and sections of 5 mice were sacrificed 1 4 and 6 days after injection The other major group received 0 5 ml NS and was examined like the first group Blood was taken daily from the infra orbital plexus of veins in each animal From 20 mice additional blood samples were taken 3 and 6 hrs after injection Procedures included WBC and differential white cell counts

The animals were sacrificed by cervical dislocation after a final blood sample and body weight measurement The thymus and spleen the mesenteric lymph node one axillary lymph node and one inguinal lymph node from each side were removed and immediately transferred to chilled Hanks solution The organs were weighed and part of the organs were taken to Lillies fixative and glutaraldehyde then embedded in paraffin and epon and sectioned in 6 and 1 micron slices The former were stained in H E the latter in Toluidine blue.

As soon as weight measurements were finished and tissue parts removed for microscopy single cell suspensions in Hanks solution were prepared from the thymus the mesenteric node and pooled axillary and inguinal nodes by aid of scissors on siliconed glass at 4 C The suspensions were diluted to get cell numbers of about 10^4/mm³ convenient for counts in a Turk counting chamber When suspensions were ready two drops of cell suspension were mixed with two drops of 0 1 per cent Nigrosin dye in 0 9 per cent NaCl—still at 4 C—and one minut later brought to the counting chamber with a capillary tube

The total number of cells was estimated by phase contrast microscopy the number of stained non viable cells by ordinary light microscopy 4 to 5 minutes after nigrosin was added to the cells. These numbers then yielded the percentage of non viable cells Since the percentage of stained cells varied between 5 and 20 a total of 500–000 cells of each organ had to be counted to give 100 coloured cells

In order to estimate flow and cell number a thoracic duct fistula was established according to *Shrewsbury* (24) in a couple of mice from each group From flow and cell number during the first hour—counted as a WBC—cells per hour were estimated

RESULTS

Blood Lymphocyte Countings

Fig 1 shows the usual drop in blood lymphocytes after use of total serum The drop is especially evident when compared to the initial rise after injection of NS By examination of Romanowsky stained blood smears, lymphocytes with twin nuclei and blastforms of lymphocytes with diameter ranging from 40 to 50 my were occasionally found in smears from the 24 hr group of ATS-treated mice—and only there

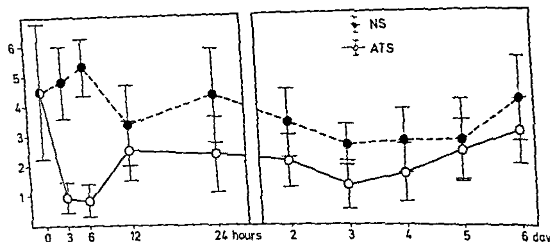


Fig 1 Variations in blood lymphocytes following a single injection of 0 5 ml antithymocytic (ATS) normal rabbit serum (NS) Ordinate Lymphocytes/1000 per mm² peripheral blood Abscissa Time hours and days

Thoracic Duct Lymphocytes

The output per hr remained normal—0 5 to 1 5 × 10⁶—for ATS—as well as NS treated mice No blastlike lymphocytes were seen in this case

Dye Exclusion Test

Fig 2 shows the variations in the number of free stained lymphoid cells in percentage of the total number of free lymphoid cells in three suspensions from each animal Each column represents a group of ten animals and bars represent standard deviation of each group Following injection of 0 5 ml ATS or 0 5 ml NS at time 0 the percentage of stained cells in suspensions from the thymus stays at control level while the percentage in suspensions from mesenteric and axillary/inguinal nodes increases to the double value in the 1–2 days group of ATS treated animals The percentage remains high also in the 4–5 days group As the table shows the percentage of stained cells in suspensions from animals treated with NS stays at control level

In preliminary investigations (unpublished) the percentage of stained cells in suspensions from thymi was found to be at normal level 3 6 12 and 18 hrs as well as 12 and 18 days after injection In the same investigations the percentage in the two other sus-

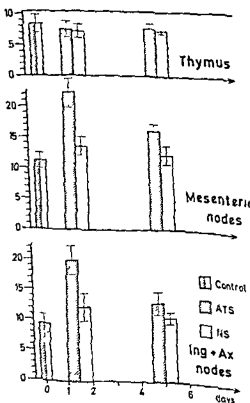


Fig 2 Change in number of non viable cells single-cell suspensions from thymus mesenteric mixed axillary/inguinal nodes from mice with 0 5 ml of antithymocytic serum (ATS) normal rabbit serum (NS) Control = untreated mice Ordinate = Percentage stained cells Abscissa Time in days after injection

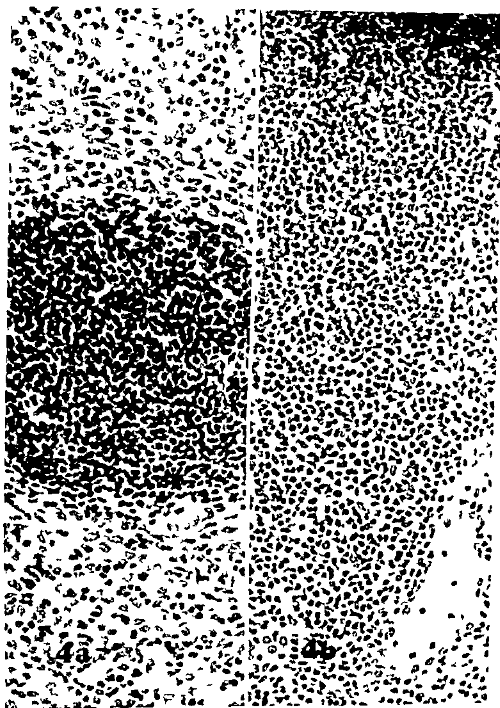


Fig 4 Photomicrographs of 5-my sections of mesenteric lymph nodes 4a 24 hr after injection of ATS 4b 24 hr after injection of NS Note the sharp limitation of the cortical nodule in 4a and the depletion of the paracortical area in a 4a compared to 4b H E. × 250

in the output from the thoracic duct Recently *Tyler et al* found a drop to ⅓ in the thoracic duct output in rats 24 hr after injection of ATS (26)

The appearance of blast like lymphoid cells in the peripheral blood after ATS treatment, noted by *Levey* & *Medawar* (17), is inconstant in the present study According to *Tyler et al* (26) they may be transformed long lived small lymphocytes that escaped destruction Still, *Monaco et al* (21) found immature large cells with approximately the same frequency in NS and ALS treated mice Peripheral lymphocytes with twin nuclei found *in vivo* after ATS treatment have not been observed in mice before but have been reported to occur after *in vitro* stimulation of lymphocyte cultures with ALS (22) The initial decrease in peripheral blood lymphocytes is well known and supports the assumption that ATS acts by destruction of long lived small lymphocytes A lack of sustained lymphopenia and even a lack of the initial drop would be insignificant in this respect since bone marrow—unaffected by ATS—feeds short lived cells into the blood (10) and thus repopulates the blood by increasing the number of short lived cells (7)

There is much variation in the reports on histological changes of the thymolymphatic system after injection of ALS Undoubtely this is due to differences in immunization schedule variability in time of examination after injection or differences in specificity of sera —whether ATS ALS raised from lymph nodes or thoracic duct lymphocytes and purified IgG have been used *Levey* & *Medawar* (17) reported that a morphologically normal thymus and moderate lymphocytic depletion of lymphoid organs would occur after injection of ATS In contrast *Martin* & *Miller* (20) found diffuse hyperplasia with dense populations of cells in the cortex and prominent germinal centres of lymph nodes from mice killed 3 days after administration of ATS On the other hand mice which from birth had been treated continuously with ATS showed 30 days old lymph nodes of an extremely alymphocytic appearance and void of germinal centres or follicles Here the thymus underwent architectural changes that extinguished the cortical medullary junction to a varied extent (19)

The abovementioned works concentrate on the morphology of longtime treatment Early changes i e structural changes following a single injection are less clarified Inclusion of histological changes found in this study however adds support to the assumption that the first change of lymph nodes is an accumulation of lymphocytes in follicles and perhaps the cortex with a simultaneous depletion of the paracortical area, without increase in the number of mitoses or pyknoses *Tyler et al* (26) reported similar findings in rats In contrast with the present study, however they also found increased numbers of lymphocytes within and around the postcapillary venules 24 hr after injection while this study shows a decreased number of lymphocytes in venules during the first hr in accordance with the postulated inhibited migration of thymus derived cells to lymphoid tissues after injection of ATS (5) The present weight constancy of lymphoid organs following ATS treatment appears unessential since the cited authors (26) in spite of weight constancy find a decreased DNA content per mg of lymphoid tissue 24 hr after ATS injection

No depletion of lymphoid organs occurs in the weeks after ATS injection—only a normalization The depletion found by the abovementioned authors is possibly due to their more chronic treatments The depletion of small lymphocytes from the marrow of thymus 24 hr after ATS injection may be interpreted as a decreased migration from the cortex (18) The otherwise natural morphology is quite comparable to findings by the abovementioned authors

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Thanks are rendered to dr *Mogens Claesson* for help with the preparation of the ATS and his interest during the work, and to dr *Niels Young* who performed the transplantations

natant after the last washing was about 1 per cent of that bound to the yeast cells Suspension without aggregation of the cells were readily obtained The *radioactivity* was measured in a γ scintillation counter with a NaI-crystal (A S Selektronik Copenhagen Denmark) The activity varied linearly with the number of yeast cells counted in Burker s counting chamber

The activity loss during 120 min varied from 2 to 5 per cent irrespective of the serum concentration used in the experiment

Serum Rabbit serum was prepared and *immediately* stored at —80 until u e

RESULTS

Experimental Conditions and Assessment of Phagocytosis

Polymorphonuclear cells from a rabbit were attached to the number of dishes necessary for each experiment A suspension of 50 × 10⁶ ⁵¹Cr labelled yeast cells suspended in 5 ml medium was poured into the dishes All the experiments were performed on one and the same preparation of yeast cells The activity *in the suspensions* was *checked to ensure that* an equal number of yeast cells was added to each dish The dishes were placed at 37° C in a termostat supplied with CO₂ keeping pH about 7 2 After 10 30, 60 and 120 minutes respectively, 2 filters were removed from each dish with tweezers and rinsed by drawing the filters ten times back and through in 3 dishes with MEM Staining and microscopic examination showed that unphagocytosed yeast cells were removed by this rinsing The activity obtained from a filter was taken as a measure the number of phagocytosed particles In control experiments without leucocytes the filters showed no activity

Reproducibility of the Method

Fig 1 gives the results of an experiment where phagocytosis was measured after 10 30 60 and 120 minutes in 6 separate dishes containing identical suspensions of yeast cells and the same number of polymorphonucleated cells In the figure each point denotes the mean activity mea ured in cp 100 sec from 2 filters taken at the same interval from one dish From Fig 1 it is obvious that good agreement was obtained between the 6 dishes. Extensive phagocytosis was found particularly during the first 30 minutes

Four experiments with all together 21 dishes showed no significant systematic difference in the activity of filters taken from different dishes and treated in the same way *

To further evaluate this method experiments were performed in the presence of some factors known to influence phagocytic activity

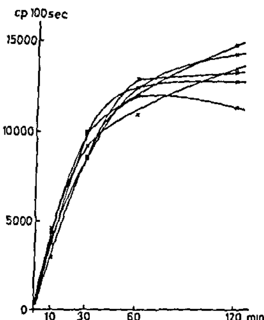


Fig 1 Phagocytosis measured in six dishes in the same way To 6 different dishes was added 15 × 10⁶ polymorphonucleated cells which adhered to 8 cellulose filter A su pension of 50 × 10⁶ Cr labelled yeast cells with 5 per cent rabbit serum was added After 10 30 60 and 120 minutes re pectively 2 filters were removed from each dish and the activity was measured in cp 100 sec Each *cross denotes* mean of two filters in the same dish Every curve denotes one dish S d of these values 9 per cent

* The statistical methods used were analysis of variance and simultaneous comparisons (7) The logarithms of the values measured were used Differences are called significant if $P < 0.05$ The analysis was done by ass *P Forsberk* at the Math. Stat Institute at the *University of Lund* with grants from Swedish Council of Medical Research Stockholm Sweden

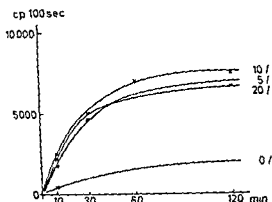


Fig 2 Phagocytosis with varying concentration of serum Leucocytes and yeast cells as in Fig 1 Explanation to symbols see Fig 1 Phagocytic activity studied in the presence of 5 10 and 20 per cent serum and without serum No significant difference between dishes containing 5 10 and 20 per cent serum The difference between these and the dish with 0 per cent serum was significant

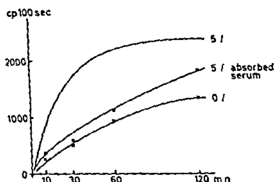


Fig 3 Phagocytosis with serum absorbed with yeast cells compared with phagocytosis with and without addition of serum Leucocytes and yeast cells as in Fig 1 Explanation to symbols see Fig 1 The absorption was done by adding 0 5 ml packed yeast cells to 1 ml serum The mixture was allowed to stand at 20 for 15 minutes In the figure is given the phagocytic activity in presence of 5 per cent unabsorbed serum 5 per cent absorbed serum and without serum Phagocytosis was significantly better in unabsorbed than in absorbed serum The difference between phagocytosis with absorbed serum and without serum is just significant on the level of P = 0 05

Serum Concentration

Phagocytic activity was studied in the presence of 5 10 and 20 per cent serum and without serum Leucocytes and serum from the same rabbit were used Medium containing 5 per cent serum improves phagocytosis significantly compared with medium without serum The results of one experiment are given in Fig 2 In this experiment as in several others addition of 10 per cent serum promoted phagocytosis somewhat more than did a concentration of 5 per cent serum but the differences was not significant Raising the concentration of the serum to 20 per cent did not further increase phagocytosis

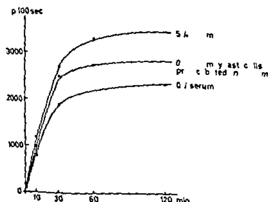


Fig 4 Phagocytosis with yeast cells incubated in serum Leucocytes and yeast cells as in Fig 1 Explanation to symbols see Fig 1 50 × 10⁶ yeast cells were incubated in 2 ml of serum at 37 C for 15 minutes washed twice in NaCl suspended in MEM and added to leucocytes without serum The phagocytosis was compared with that recorded with untreated cells in MEM containing 5 per cent and 0 per cent serum The incubated yeast cells were phagocytosed significantly more than untreated cells in serum free environments but significantly less than untreated cells in the presence of 5 per cent serum

Absorption of Serum with Yeast Cells

Phagocytosis was more marked in the presence of unabsorbed serum than of absorbed serum The degree of phagocytosis with absorbed serum was however higher than in the absence of serum (Fig 3)

These results suggested that in the rabbit serum contained opsonins that could be absorbed out by yeast cells In order further to test this possibility yeast cells were incubated in serum and washed The incubated yeast

E coli and protein coated betonite particles labelled with ¹²⁵I He also reported a considerable loss of ¹²⁵I, particularly from the bentonite particles owing to both serum and cell bound factors In a later study (6) these difficulties were overcome by treatment of the bentonite particles with carbodiimide No signs of raised release of ⁵¹Cr from yeast cells in the presence of serum was found with the system used in the present study The slight release of ⁵¹Cr, varying between 2–5 per cent, was independent of the addition of serum

The consequence of having the phagocytosing cells in suspension or adhered to a surface for the calculation of phagocytosis has been discussed (4) No essential difference in the phagocytosing ability of the cells has been reported There are however technical advantages of using adherent cells as reported by Trippestad and Midvedt who used polymorphonucleated leucocytes attached to glass (13) It is easy to wash off undesired cells such as erythrocytes and lymphocytes Moreover it is easy to wash off unphagocytosed particles

In order to asses the usefulness of the method described it was tried in the presence of some factors known to be capable of affecting phagocytic activity (1 4 5 9 12 13 14) The results reported showed good agreement with earlier findings and elucidate the value of the method It can be noted that like *Maaloe* and *Tullis* & *Surgenor* (9 14) but unlike *Trippestad* & *Midvedt* (13) we found that addition of serum heated at 56° 30 inhibited phagocytic activity

The results obtained with the method described suggest that it should be useful in the analysis of the phagocytosis process

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I wish to thank Mrs. *E Miller* for skilful technical assistance

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BRIEF REPORT

DEMONSTRATION BY DISC ELECTROPHORESIS OF THE LIPOPROTEIN CARRYING THE Lp(a) ANTIGEN IN HUMAN SERA

Henrik Garoff Kai Simons Christian Ehnholm and *Kare Berg*

In 1963 *Berg* described a genetic polymorphism in the human serum β lipoproteins Two phenotypes Lp(a+) and Lp(a −) could be distinguished by immunodiffusion with rabbit antiserum *Wiegandt et al* (1968) showed that the Lp(a+) activity was found in a lipoprotein fraction with a higher density 1 050 – 1 125 g/ml than the bulk of the β lipoproteins In polyacrylamide gel electrophoresis this serum fraction gave three bands a rapidly migrating α lipoprotein band a slowly migrating β lipoprotein band and a very slow band containing the Lp(a) lipoprotein. The corresponding fraction of the sera of Lp(a—) individuals lacked the Lp(a) lipoprotein band in electrophoresis *Utermann & Wiegandt* (1969) isolated an electrophoretically pure Lp(a) lipoprotein This contained less lipid than the bulk of the β lipoproteins but shared antigenic determinants with them We have confirmed their findings (*Simons & Ehnholm* to be published)

The immunological method of *Berg* (1964b) has thus far been the only one by which to determine Lp types In this report we describe a disc electrophoresis technique which may become useful for Lp typing

Materials and Methods

Human Sera Non lipaemic fresh sera from 61 healthy Finns men and women were used.

Immunological method Lipoproteins with densities from 1 050 to 1 125 g/ml were separated from the serum of Lp(a+) individuals by ultracentrifugation as described by *Havel et al* (1955) using a Ti 60 rotor in a Beckman model L4 ultracentrifuge They were then used to immunize rabbits Otherwise the anti Lp(a) antiserum was prepared according to *Berg* (1965) Lp typing was performed as previously described (*Berg* 1964b)

Disc electrophoretic method One day before electrophoresis the sera were stained with Sudan black B in ethylene glycol solution prepared according to *McDonald & Ribeiro* (1959) in 9 parts serum to one part stain Before electrophoresis sucrose to a final concentration of 6 per cent was added to the mixture. The disc electrophoresis system we used has been described by *Natajan et al* (1966) The concentrating gel contained 2 5 per cent acrylamide and the separating gel 3 75 per cent. The prestained sera 20 or 40 µl were layered on top of the concentrating gels in tubes filled with Tris-glycine buffer of pH 8 4 Electrophoresis was performed at 2 5 mA per tube for 110 minutes

The bands were poorly separated if we used more than 40 µl of prestained serum If we used less than 20 µl the Lp(a) lipoprotein bands were usually very weak and the results hard to interpret Prestaining of the samples with Sudan black B gave clearer results than Oil Red O staining of the gels after electrophoresis

Results and Discussion

Out of the 61 sera tested for Lp(a) activity by double diffusion in agar gel 19 were Lp(a+) and 42 Lp(a—) Thus the frequencies of the phenotypes Lp(a+) and Lp(a—) (31 per cent and 69 per cent respectively) were not significantly different from those reported in the case of Norwegians (*Berg & Mohr* 1963) In Fig 1 the electrophoretic patterns of an Lp(a+) serum (A and B) and Lp(a—) serum (C) are shown Lp(a+) sera gave four bands the α lipoprotein band (not shown) the main β lipoprotein band the Lp(a) lipoprotein band and material (very low density lipoproteins) that barely entered the separating gel The latter material was not seen in the isolated serum fraction of 1 050–1 125 g/ml (Fig 1D)

The slow band which is here referred to as *the* Lp(a) lipoprotein band appeared between the origin and the main β lipoprotein zone in Lp(a+) sera It was lacking in sera from Lp(a—) individuals Some of the Lp(a+) sera gave more intensely stained bands than others and this difference seemed to correlate with the stren...

Received 8 ii 70 from the *Department of Serology and Bacteriology University of Helsinki Helsinki Finland and Institute of Medical Genetics University of Oslo Oslo Norway*

Neither the results of the transformation experiments nor the mole per cent G + C serve as evidence for the inclusion of the organism in the genus *Moraxella*. On the other hand the inclusion cannot be definitely rejected on the basis of these observations. The mole per cent G + C is not more than 2-3 per cent higher than in *M. osloensis* and one of the recognized *Moraxella* species *M. phenylpyrouvica* (4) also appears incompatible with *M. nonliquefaciens* in streptomycin resistance transformations and only shows very slight affinity to *M. osloensis*.

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A SIMPLE TEST FOR PEROXIDE SECRETION BY *MYCOPLASMA*

Klaus Lind

It was observed that erythrocytes from guinea pig and man which had haemadsorbed (3) to colonies of *Mycoplasma (M.) pneumoniae* or *M. gallisepticum* stained with methylene blue (MB) when this dye was added to the agar plate. The staining which increased within a few minutes to a deep blue colour occurred only in erythrocytes that were in contact with colonies (Fig. 1).

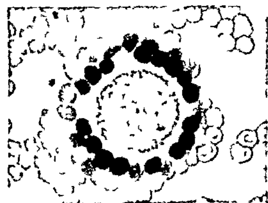


Fig. 1. Methylene blue staining of erythrocytes induced by a colony of *M. pneumoniae*.

Received 20.iv.70. *from the Department of Toxoplasmosis and Viral Diseases, Statens Seruminstitut, Copenhagen, Denmark.*

Preliminary investigations of this phenomenon are presented.

Material and Methods

Mycoplasmas: The strains indicated in Table 1 were grown on standard mycoplasma agar medium (1). From inoculated agar plates blocks that contained less than 25 colonies per square cm were cut out and placed on microscope slides.

Erythrocytes: Blood from guinea pig and man (group 0I) was collected in citric acid-sodium citrate-glucose anticoagulant. The erythrocytes were washed twice and then 0.5 and 5 per cent suspensions were made in phosphate buffered saline pH 7.2 (PBS).

Reagents: Methylene blue (MB) (Chroma Gesellschaft, Stuttgart) was used as 0.1 and 0.01 per cent solutions in PBS. Peroxidase (Sigma Cat. no. P8250, Type II) was dissolved in PBS (1000 PU/ml). Merthiolate was applied as a 1:5000 solution in PBS. Neuraminidase was the receptor destroying enzyme from *V. cholerae* (Behringwerke).

Tests for methylene blue staining of erythrocytes (MBS) and haemadsorption (HAD) (3): One drop of the 0.5 per cent erythrocyte suspension and one drop of MB solution were placed on each agar block with mycoplasma colonies and allowed to react at room temperature for 10 minutes. The

TABLE 1 *Induction of Methylene Blue Staining of Guinea Pig Erythrocytes (MBS) Haemolysis (HL) and Haemadsorption (HAD) by Various Mycoplasma Species*

Species	Strains	MBS	HL	HAD
M pneumoniae	Mac Mp 8	+	+	+
M gallisepticum	X95 S6	+	+	+
M canis	PG 14	+	+	+
M edwardii	EF 22	+	+	(+)
M mycoides var myc	BP	+	+	0
M hyorhinis	EF 25	+	+	0
M fermentans	EF 9	+	+	0
M laidlawii	EF 22	+	+	0
M arthritidis	S 16	+	+	0
M spumans	PG 13	0	0	+
M orale type 1	Symons	0	0	0
M salivarium	PG 20	0	0	0

blocks ere observed in the microscope before and after washing with PBS

Haemolysis produced by mycoplasma colonies was observed on agar blocks by spreading a drop of 5 per cent erythrocytes over the agar The preparations were incubated overnight in a humid atmosphere at 37 C The zones of haemolysis were measured microscopically

Results and Discussion

The MBS was induced only by mycoplasmas which also haemolysed the red cells (Table 1) For *M arthritidis* the concentration of MB was critical While 0 01 per cent MB readily stained the red cells that were in contact with the colonies this did not happen with 0 1 per cent MB under the same conditions The Table also sho s that MBS was independent of haemadsorption Erythrocytes which accidentally were in contact for a few minutes with colonies of some non haemadsorbing species were stained by the dye and retained the colour even if removed from the colony Correspondingly it was ob erved that erythrocytes which had been treated with neuraminidase and hence were not haemadsorbed were stained intensely when in contact with colonies of *e g M gallisepticum* and *M pneumoniae*

The MBS was induced also by colonies of *M pneumoniae* and *M gallisepticum* grown on glass and washed in PBS as well as by mycoplasma cells and microcolonies of these species grown in broth

The possibility was considered that the MBS might be dependent on and related to the liberation of peroxide from mycoplasmas (5) This was confirmed by the inhibition of MBS of colony adsorbed red cells by peroxidase This enzym also inhibited the haemoly produced by the same mycoplasmas The MBS was furth rmore inhibited by merthiolate wh ch did not inhibit the haem adsorption *M laidla* (I F22) induced a ery in tense MBS which however was not reduced by the concentration of peroxidase that inhibited the haemolysis

Another possibility is that the MBS may be correlated to the reduction of methaemoglobin (discussed in 4) This is formed by the action of *M gallisepticum* (5) and probably also by *M pneumoniae* (author s observation)

According to *Sass et al* (4) normal erythrocytes have the ability to accumulate MB which they transform to leukoMB and retain Erythrocytes can accompli h at least a 150–200 fold concentration from a medium containing 0 013 mM MB The maintenance of MB in the reduced state within normal red cells is supposed to be a result of enzyme functions despite the presence of oxygen

It is suggested that the MBS observed might be due to an interaction between such enzyme functions and the high concentration of peroxide created near the mycoplasmas In case of peroxide secreting mycoplasmas that are sensitive to MB (2) the MBS may only take place provided the concentration of MB applied is below that which is toxic to the secretory activity

The tentative *conclusion* is that the MBS may serve as a rapid and simple test for peroxide secretion by mycoplasmas

1 A ta p h m b l d S B 8

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SEPARATION FROM HeLa CELL CULTURES OF THREE ESTERASES ONE OF WHICH RESEMBLES HUMAN ACTIVATED C1s

Gerolf von Zeipel

The anticomplementary activity of serumfree fluids of non infected HeLa cell cultures (6) was recently (7) found to be correlated to esterase activity In the present study this activity is shown to be complex, three esterases being found so far One of these splits a series of esters in a manner similar to human activated C1s and also has the ability of the latter to destroy the hemolytic activity of C4

Materials and Methods

HeLa cells were cultured and harvested fluids processed as described earlier (6 7)

However in one experiment HeLa cells were *kept under special conditions These cells* were grown out in the usual medium (6) plastic bottles (Falcon U S A) being used instead of glass bottles The cultures were then maintained for three months in the initial bottles with the serumfree medium of *Neuman & Tytell* (5) The cell sheet was confluent and in good condition throughout *the experiment* The number of detached cells present in each four day harvest of medium indicated an about twofold cell multiplication at this interval Harvested fluids were processed as referred to above

Human activated C1s One preparation ($\overline{C1s}$ La) was from Professor *Inna Brita Laurell* University of Lund Sweden another ($\overline{C1s}$ Ic) was from Dr *J Pensky* Case Western Reserve University Cleveland Ohio U S A Both preparations had been purified according to the first two steps of the method of *Haines & Lepow* (3) C1s Pe in addition on hydroxylapatite

Chromatography was performed on hydroxylapatite (Serva Germany) and on DEAE Sephadex A 25 (*Pharmacia Sweden*) Columns for primary runs were 20 25 × 2 5 cm and loaded with 300–400 mg of protein For further purification material from a primary column was run through a 20–25 × 1 5 *cm column* which was loaded with 60–80 mg of protein Hydroxylapatite columns were *eluted* by linear gradient phosphate buffers (1500 ml/run) *ranging from* 0 01 *to* 0 7 M *at* pH 6 87 DEAE Sephadex columns were eluted by linear NaCl gradients (1500 ml for large 800 ml for small columns) ranging from 0 to 0 7 M in *tris buffer* of an ionic strength of 0 025 and pH 8

Gelfiltration on Sephadex G 200 (Pharmacia Sweden) was carried out in columns 90 × 2 5 cm They were loaded with 5 ml amounts of material Elution occurred at a flow rate of 24 ml/hr with the following buffer of pH 7 3 tris 0 05 M HCl 0 04 M *and* NaCl 0 098 M

Protein measurements were made with the Folin phenol reagent (4)

Esterase activity was assayed as described earlier (7) *Preparations* were tested after equilibration against a 0 005 M phosphate buffer (pH 7 5) with 0 15 M NaCl

Inactivation of C4 was tested by the simplified assay of *Haines & Lepow* (3)

Results

Chromatography The esterase activity of HeLa cell fluids (H) was resolved by chromatography into three enzyme fractions H 1 H 2 and H 3 These differed from each other as to hydrolysis of various synthetic amino acid esters (see Table 1)

The main peak of each enzyme *fraction* was eluted from hydroxylapatite at the following range of buffer concentrations H 1 0 02 0 074 M H 2 0 095 0 22 M H 3 0 23 0 31 M The recovery of each fraction was *40 to 50 per cent*

The enzyme fractions were eluted from DEAE Sephadex in the reversed order H 3 0 03 0 14 M H 2 0 17–0 24 M However the recovery of *H 1* having a peak at 0 27–0 31 M was only 4 to 7 per cent in contrast to a recovery *of 40 to 50 per cent* for the two other fractions

The effluent from Sephadex G 200 was analysed for H 1 and H 2 only *There was no separation* between these esterases but the yield was *different* The recovery of H 2 was about 30 per cent As on

Received 21 1 70 he [illegible] *Department of the Central P[illegible] ory of Stockholm* [illegible] *Stockholm* St

TABLE 1 *Hydrolysis of Synthetic Amino Acid Esters*

Substrate	Enzyme					
	H	H 2 D S	C1s La	C1s Pe	H 1	H 3
A N acetyl L arginine Me HCl[1]	111	110	141	140	–	11
B N α benzoyl L-arginine Ee HCl	34	34	45	42		5
C N α CBZ L arginine Me HCl[1]	73	81	85	92	–	12
D p tosyl L-arginine Me HCl[3]	22	36	37	34	–	–
E N α acetyl L-lysine Me HCl[1]	261*	291*	293*	341*	3	35
F N α acetylglycyl L-lysine Me acetat[1]	410*	406*	409*	483*	–	29
G N α CBZ L lysine Me HCl[1]	123	115	132	134	3	
H α tosyl L lysine Me HCl[3]	44	52	47	53	–	
I N acetyl L phenylalanine Ee[2]	167	12	12	16	100 (13)	33
J N acetyl L-tyrosine Ee[4]	100 (5 9)	100 (8 4)	100 (11)	100 (7 9)	10	36
K N acetyl L tryptophan Ee[5]	55	16	17	12	–	100 (5 1)

Me = methyl ester Ee = ethyl ester – = no hydrolysis – not done

[1] Cyclo U S A [2] Sigma U S A [3] Calbiochem Switzerland [4] Brit. Drug House England [5] Mann U S A

Numbers indicate rates of hydrolysis of esters (A to K) relative to the reference ester These are J for enzymes H H 2 D S C1s La and C1s Pe I for H 1 and K for H 3 The reference ester has number 100 and within parantheses amount in micromoles of H liberated in 1 hr by the amount of enzyme in a test volume of 2 5 ml * at number indicate rate of hydrolysis between ester and reference ester at measurements for 30 minutes

DEAE Sephadex however most of fraction H 1 was not eluted under the conditions of the test This may indicate an interaction with Sephadex

The enzyme fractions referred to in the Table as well as in the tests on C4 were as follows

H = starting material H 1 = first peak material from hydroxylapatite activity on N acetyl L-phenylalanine ethyl ester 7 6 micromoles of H liberated in one hr per mg of protein H 2 DS = second peak material from hydroxylapatite further purified on DEAE-Sephadex activity on N acetyl L tyrosine ethyl ester 9 7 micromoles of H liberated in one hr per mg of protein H 3 = third peak material from hydroxylapatite activity on N acetyl L tryptophan ethyl ester 4 5 micromoles of H liberated in one hr per mg of protein

C4 tests Inactivation of C4 by preparations H 2 DS C1s La and C1s Pe was of a similar order when referred to the same activity upon N acetyl L-tyrosine ethyl ester On the other hand H 1 and H 3 lacked any effect at the concentrations tested

Synthesis of esterases by HeLa cells HeLa cell cultures maintained for three months in serumfree medium produced at an undiminished rate the two esterases referred to above as H 1 and H 2 H 3 not being tested From N acetyl-L-phenylalanine ethyl ester indicator of H 1 194 and 179 micromoles of H were liberated by the total amount of enzymes produced during the first and latter 6 week period respectively The corresponding figures for the hydrolysis of N acetyl L-tyrosine ethyl ester indicator of H 2 were 64 and 66 micromoles of H during the same periods Although the esters are not specific for H 1 and H 2 respectively the rates of hydrolysis obtained indicate the presence of both esterases (see Table 1) The continuous production in *serumfree HeLa* cell cultures of the esterases in question indicates that these were really synthesized by the cells and excludes the possibility that they somehow originated from the serum of the outgrowth medium.

Discussion

As shown in the Table enzyme H 2 DS and human activated C1s have similar esterolytic properties This together with a shared ability to inactivate C4 suggests a close relationship between these enzymes It should however be pointed out that H 2 DS although subjected to similar chromatographic separation steps was a less pure preparation than the reference samples of human activated C1s which were about 30 times more active per mg of prot [illegible] would therefore be desirable to d [illegible] replace [illegible] ity of H 2 DS to [illegible] te forma on o [illegible]

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T Holme S Arvidsson & T Wadstrom Department of Bacteriology Karolinska Institutet Stockholm FORMATION OF EXTRACELLULAR TOXINS AND ENZYMES IN CONTINUOUS CULTURE OF *STAPHYLOCOCCUS AUREUS*

J Gen Microbiol 1970 In press

Blanka Rutberg Department of Bacteriology Karolinska Institutet Stockholm CITRIC ACID CYCLE MUTANTS OF BACILLUS SUBTILIS

J Bacteriol 1970 In press

S E Holm & A J R Moller Department of Bacteriology Institute of Medical Microbiology University of Gothenburg SOME FACTORS OF IMPORTANCE FOR THE PRODUCTION OF STREPTOLYSIN O

Growth media developed by the authors were used for studying the production of streptolysin O under various conditions The correlation between the antigenicity and the haemolytic activity after storage at different temperatures for various times was also studied

The optimal pH range for streptolysin O production with the growth medium employed was 6 2–6 8 The high concentration of glucose (1 per cent) which is essential for good growth and streptolysin O production can cause a rapid pH decrease which necessitates a continuous pH adjustment during the whole period of cultivation At a constant pH of 6 8 streptolysin O activity was demonstrated after 6 hours cultivation and reached a maximal value after a further 6 hours provided the glucose concentration was maintained at 1 per cent in the growth medium The storeability was largely depending on the pH at which the streptolysin was produced The activity of streptolysin O produced at pH 6 8 did not decrease significantly at 37 C during 24 hours and streptolysin could be stored for more than two years at +4 C without detectable loss of haemolytic activity or antigenicity

G Bergqvist & B Hurvell Department of Pediatrics Crown Princess Lovisas Children Hospital and the National Veterinary Institute Stockholm NEONATAL INFECTIONS CAUSED BY STREPTOCOCCUS AGALACTIAE (LANCEFIELD GROUP B)

Infections caused by Streptococci Group B are of importance in veterinary practice as they are a cause of mastitis in cows Reports concerning the isolation of B group streptococci in man have hitherto been relatively rare In adults such infections have become manifest as urinary infections, puerperal sepsis endocarditis arthritis osteomyelitis and wound infections especially among diabetics and in neonates as septicaemia meningitis and omphalitis Some authors report the occurrence of B-streptococci in the vagina of pregnant women in 5 per cent of investigated cases

Many neonatal infections caused by Streptococcus group B have been reported from the USA and Europe whereas only a few cases have been described to date from Scandinavia

During the last year 5 cases of this type of infection have been treated at the Crown Princess Lovisas Children s Hospital Several further cases from the Stockholm area during the past year are known The same group of streptococci was isolated from the vaginas of 4 of the mothers Four of the neonates had severe infections and two of them died

To estimate the significance and frequency of this infection in neonates a study of the occurrence of Streptococci group B among pregnant women at term has been started

G Lindbom & G Laurell Department of Clin Bacteriology The Institute of Medical Microbiology Uppsala STAPHYLOCOCCAL INFECTIONS IN A THORACIC SURGICAL UNIT AIRBORNE SPREAD OR NOT?

Scand J Inf Dis 1970 In press

G Wallmark Agneta Magnusson & B Tornberg Departments of Bacteriology and Pathology Sodersjukhuset Stockholm GENERALIZED STAPH AUREUS INFECTION FOUND AT AUTOPSY

The incidence of generalized infection by *Staph aureus* has been determined on a non selected 1 year autopsy series of 1?06 cases The samples for culture were taken immediately after opening the body from liver spleen kidney and lungs by means of a swab introduced into the tissues after burning the surface of the organs *in situ* In 68 cases (5 6 per cent) *Staph aureus* was found in 2 or usually more organs the phage type being identical in the different organs the growth mostly profuse and phage type 52/52A/80 81 predominating Various debilitating diseases were diagnosed clinically and at autopsy in most instances Thirty-eight patients had been in a medical ward and 27 in a surgical Clinically septicaemia was suspected in only 9 patients with a positive blood culture in 4 Histologically the lungs showed acute focal pneumonia in 54 cases with abscesses in ?1 in addition microabscesses were found in different organs in 11 instances myocardium and kidney predominating

but also the liver pancreas and bone marrow might be affected The sampling technique used proved to give reliable results as shown by the bacteriological pattern and the histopathological findings

E Nordenfelt Institute of Medical Microbiology Lund PRESENCE OF AUSTRALIA ANTIGEN IN A HEPATITIS EPIDEMIC AMONG PATIENTS TREATED WITH DIALYSIS

During the summer of 1968 several cases of hepatitis appeared among the patients in the dialysis ward Med Clin B Lund Since Oct 1968 all patients have been followed by blood samples every fortnight and tested for presence of Australia (Au) antigen From Oct 1968 Oct 1969 29 patients have been studied among whom 24 contracted hepatitis Practically 100 per cent correlation was found between presence of Au antigen and hepatitis The antigen was detected up to 6 weeks before clinical signs of hepatitis were manifest Patients treated with dialysis seem to become chronic carriers of Au antigen several have been found to be carriers for more than year This is in sharp contrast with findings among hepatitis patients without complicating disorders in whom the Au antigen appears transiently at the beginning of the disease

J Kjellander & G Lindé Dept of clinical Bacteriology Regional Hospital Örebro A COMPARISON BETWEEN CHEMICAL AND BACTERIOLOGICAL TESTS FOR BACTERURIA

992 urine specimens were tested with the chemical and bacteriological tests for bacteruria available on the Swedish market All specimens were cultured for comparison with the calibrated loop technique

238 specimens contained more than 100 000 organisms per ml according to the standard loop culture Among these 67 per cent were positive with the TTC test (Uroscreen®) 55 per cent with the glucose test (Uriglox®) 45 per cent with one nitrite test (Urnitest®) and 37 per cent with an other (BM nitrite®) The proportion of false positives were 5 per cent 1 per cent 0 per cent and 0 per cent respectively There was good correlation between the standard loop culture and the two agar dip slide tests (Uricult® and Inoculator®)

There was a marked difference in the outcome of the chemical tests in specimens with low and high counts For instance the TTC test was positive in 72 per cent of the specimens containing 10^5 10^6 organisms per ml and in 92 per cent of the specimens containing 10^7–10^8 organisms per ml Similar results were obtained with the glucose test whereas the nitrite tests tended to become positive less often when the counts were high than when they were intermediate

The investigation thus showed that none of the chemical tests can replace the culture for detecting bacteruria in a clinical material It also shows the importance of taking the levels of bacteruria into consideration when different investigations in this field are to be compared

B Nyström Clinical Bacteriological Lab Karolinska Hospital Stockholm METHODS FOR MICROBIOLOGICAL TESTING OF ETHYLENE OXIDE STERILIZING IN HOSPITAL

Two makes of ethylene oxide autoclaves small models intended for hospital use have been tested None has been found satisfactory

Among various commercially available spore tests used the tests containing B subtilis vacuum dried in quartz sand available from the Serum Institute Copenhagen proved more ethylene oxide resistant than the British ones described by Beeby & Whitehouse or Swedish ones by which to test ordinary steam autoclaves

Aluminium foil strips containing dried-out Staph aureus dried onto the strips from broth proved more ethylene oxide resistant than any spore tests used in this investigation Newly made Staph aureus trips not dried-out with 10^7 organisms were somewhat more resistant than the British spore tests but less reshistant than the Danish ones

As dried out Staph aureus on a metal surface might well occur in non disposable items to be ethylene oxide sterilized in hospitals this sterilization method should not be used in hospitals at least not for items that can be sterilized in any other way If ethylene oxide sterilization is used for non disposable items in hospitals it is questioned whether spore tests or test pieces containing driedout Staph aureus should be used

H Gnarpe Department of Bacteriology Institute of Medical Microbiology University of Uppsala Uppsala EXPERIMENTAL SPHEROPLAST INFECTIONS IN THE URINARY TRACT TREATED WITH METACYCLINE

Due to the high content of electrolytes conditions for the survival of cell wall defective organisms are good in the urinary tract Therefore spheroplasts induced by penicillin treatment may survive in the urinary tract Earlier investigations have shown that they may be eliminated by a combined treat

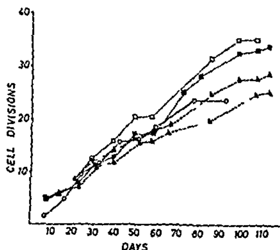


Fig 1 The effect of commercial and pure gentamicin on the growth of human embryonic diploid lung fibroblasts (○——○) control (△- - -△) commercial and (▲- - -▲) pure gentamicin at 1 µg/ml (□ □) commercial and (■ ■) pure gentamicin at 10 µg/ml

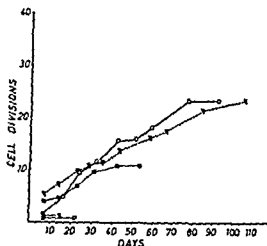


Fig 2 The effect of commercial and pure gentamicin on the growth of human embryonic diploid lung fibroblasts (○——○) control (▽- - -▽) commercial and (▼- - -▼) pure gentamicin at 100 µg/ml (□ □) commercial and (■ ■) pure gentamicin at 500 µg/ml

7 8 with 1 N NOH The suspended cells were mixed well and counted directly in the suspension An amount equivalent to 2×10^5 cells was added to a new bottle containing 20 ml Eagle s medium + 10 per cent calf serum but no gentamicin If the volume of the trypsinized cell suspension to be added was greater than 5 ml the cell suspension was centrifuged resuspended in 20 ml medium and added to the new bottle The bottles were incubated at 35 C for 6 hours after which the medium was changed with 20 ml Eagle s + 10 per cent calf serum plus the appropriate concentration of gentamicin and the number of cells which had attached to the surface was counted by a method described earlier (5) The number of cells in 8 field-of views scattered over corresponding places on the growth surface was counted with a Leitz inverted microscope The number of cells per bottle was determined by multiplying the average number of cells per field-of view by the ratio of the area of the growth surface to the area of the field-of view counting the bottles were reincubated at C The medium was changed after 5 days in tervals or when it became acid The cultures were trypsinized when the cell layer became confluent

RESULTS AND DISCUSSION

At 1 µg/ml and 10 µg/ml the commercial preparation produced a slight but consistently better growth than the pure antibiotic (Fig 1) At 1 µg/ml the growth was similar to that of the control but at 10 µg/ml the cells grew better and had a much longer growth potential (about 35 cell divisions) than the control (about 23 cell divisions) The differences between the commercial and pure gentamicin curves at 1 µg/ml and 10 µg/ml were similar suggesting that if this difference were due to the preservatives added to the commercial preparation, there would be no apparent concentration effect over this range

Fig 2 shows that the commercial preparation at 100 µg/ml and 500 µg/ml was highly toxic, whereas the corresponding concentrations of pure gentamicin permitted some growth to occur but 500 µg/ml was obviously inhibitory This latter culture became contaminated with an alpha streptococci and a yeast identified as Candida parapsilosis* and had to be discarded The growth rate with 100 µg/ml was similar to that of the control but much less than the rate with 10 µg/ml Nevertheless this culture continued to grow after all others had ceased It finally reached senescence after about 200 days in culture and a total of 34 cell divisions Thus, the

* The author appreciates the co-operation of Drs C Henning and H Pa'drol National Bacteriology Lab Sweden in identifying the contaminating micro-organisms.

TABLE 1 *The Per Cent Cells Attaching to the Glass Surface after 6 Hours Incubation*

Passage	Control	Commercial Gentamicin µg/ml				Pure Gentamicin µg/ml			
		1	10	100	500	1	10	100	500
3		100	100	100	100	100	100	100	100
4	100	100	100	100	60	100	100	100	100
5	55	100	50	17		75	100	95	90
6	42	55	38			60	75	100	55
7	34	60	55			100	60	100	100
8	47	65	70			39	39	75	47
9	100	100	100			100	100	65	7
10	100	100	0 4			70	9	95	
11	16	14	55			75	75	19	
12	100	65	100			6	32	75	
13	6	100	60			60	100	26	
14		100	44			58	70	39	
15		47				12	30	100	
16		4 3						38	

longevity was the same as with 10 µg/ml gentamicin

In almost all concentrations of gentamicin the cell attachment was at least as good and perhaps better than that in the control (Table 1) At 1 µg/ml the commercial preparation permitted better attachment than the pure gentamicin but at 10 µg/ml this relationship was reversed Concentrations of gentamicin as high as 100 µg/ml and 500 µg/ml produced no marked inhibition in the cells ability to attach to the glass Since cell adsorption involves the *interaction of the cell* and glass surfaces one can assume that gentamicin had no effect on cell wall properties or synthesis

The data suggests that one or more of the substances added as preservatives to the commercial preparation may have slight growth stimulatory activity at low concentrations but are toxic at the concentrations present in the 100 µg/ml gentamicin sample (4 5 µg/ml methyl paraben 0 5 µg/ml propyl paraben 8 µg/ml Na bisulphite and 0 25 µg/ml disodium ethylene diamine tetra acetate) The antibiotic had a marked growth promoting effect at 10 µg/ml and also increased the longevity of the diploid cells *in vitro* at a concentration of 10 µg/ml and 100 µg/ml When using the commercial garamycin preparation a dose of 10 µg/ml should not be exceeded for any length of time in tissue culture medium

The relation of these doses to tissue mass may be calculated by assuming an average final value of 10^6 cells per bottle The value of 2×10^{-9} g/cell published by *Hayflick & Moorhead* (3) gives an estimated 2 mg cells in each bottle Therefore the commercial preparation should be toxic if between 100 g to 1 kg gentamicin per kg body weight were administered to an animal Although the concentrations per weight of tissue was very high compared to clinical usage, the growth stimulatory effects observed warrants further investigation

The author gratefully acknowledges the excellent technical assistance of Mrs *Ann Bjork*

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Migration of splenic cells into the blood has been studied after administration of antigens other than sheep erythrocytes After intravenous administration of typhoid vaccine to rats, *Cannon & Wissler* (1965) demonstrated entry into the blood stream of morphologically diverse lymphoid cells which were probably formed in the spleen *Ernstrom & Sandberg* (1968) showed a migration of cells from spleen to blood in normal young guinea pigs, and this migration was found to increase after subcutaneous injection of pertussis vaccine

Histological evidence of a direct entry of spleen cells into the blood has also been presented (*Langevoort* 1963 *Pelc* 1968)

In the present investigation migration of splenic cells into the blood stream in young guinea pigs was studied after a single intraperitoneal injection of sheep red blood cells (SRBC)

MATERIAL AND METHODS

A total of 131 male guinea pigs with an initial weight of 221 ± 19 g (mean ± S D) were used 69 of them were given a single intraperitoneal injection of a sheep erythrocyte suspension (fresh erythrocytes washed four times with Claus Jensen buffer and diluted to a 10 per cent suspension in 0 9 per cent saline) in a dose of 2 5 ml per animal The injected animals were divided into 5 groups investigated 3 6 9 12 and 15 days after the injection (12 16 15 12 and 14 animals respectively) Litter mates of the animals injected with sheep erythrocytes a total of 62 animals were as controls and injected with 2 5 ml of 0 9 ent saline per animal and divided into groups (12 14 11 13 and 12 animals respectively)

At investigation the animals were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg/kg body weight i p) The peritoneal cavity was opened by an incision between the last two ribs on the left side Blood was collected in a dry heparinized pipette (Heparin® Vitrum Stockholm Sweden) after incision of a splenic vein Immediately afterwards a splenic artery was incised and arterial blood collected the same way The blood samples were used for white cell counts in Bürker counting chamber Further blood samp were used for differentiation between subclasses f lymphocytes after supravital staining of the mitochondria in the white cells Blood collected in apillary tubes was centrifugated 5 *minutes* at 9000 r p m. for haematocrit determination in order to exclude the possibility that the splenic *veno-arterial differences in* the white cell content found were due to a haemo-concentration or -dilution in the spleen Janus green B and neutral red were used for the supravital staining of the white blood cells (for details see *Ernstrom et al* 1969) and the lymphocytes were divided into six classes according to the cellular content of mitochondria cells with 0–5 6–10 11–15 16–20 21–30 and > 30 mitochondria Lymphocytes with 0–10 11–20 and > 20 mitochondria were called low MC (mitochondrial *content*) medium MC and high MC lymphocytes *respectively* According to some investigators the mitochondrial content is correlated with the size of the lymphocytes (*Wiseman* 1931 *Fichtelius & Larsson* 1961 *Ernstrom & Larsson* 1963) low MC corresponding to small lymphocytes high MC to large lymphocytes

The absolute number of lymphocytes per mm³ of blood belonging to the different categories was calculated from the counts of mononuclear cells and the distribution of lymphocytes with different MC The values obtained from splenic artery and vein blood were compared in the individual animals and the differences were analysed statistically by Student s *t* test The haematocrit values were analysed in the same way

The weight of the following organs was determined The spleen thymus cervical lymph nodes (all together) and mesenteral lymph nodes (all together)

RESULTS

Blood Cell Population

The total number of lymphocytes per mm³ of arterial blood was *not significantly affected*

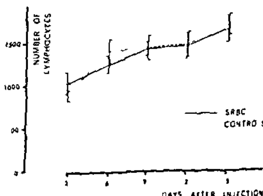


Fig 1 Total number of lymphocytes per mm³ of arterial blood at various intervals after a single injection of either sheep red blood cells (SRBC) or saline (controls) Mean ± S E

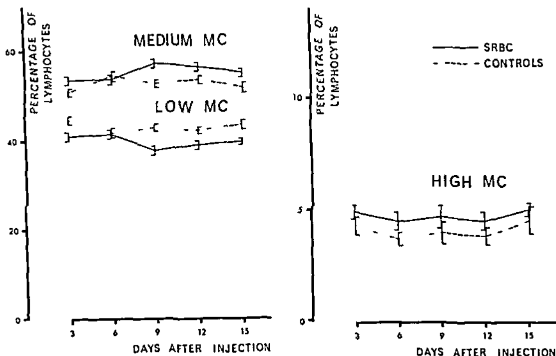


Fig 2 Percentage in splenic artery blood of lymphocytes with different mitochondrial content (MC) at different intervals after a single injection of either sheep red blood cells (SRBC) or saline (controls) Mean ± S E

by treatment with SRBC Both in the immunized and the control animals there was a steady increase in the number of blood lymphocytes from about 1 000 cells to about 1 700 cells per mm³ during the investigation period (Fig 1)

After injection with SRBC there was a change in the relative amounts of low MC medium MC and high MC lymphocytes (Fig 2) with an increased percentage of medium and large MC lymphocytes and a decreased percentage of small MC lympho cytes When studying each of the six sub groups of lymphocytes separately the most significant change was seen in the number of lymphocytes with 0–5 mitochondria per cell (Fig 3) From day 9 after SRBC injection there was an increasing deficiency of these cells in the peripheral blood when compared with that of the controls The other sub groups showed some variations that are at least statistically insignificant (see further under discussion)

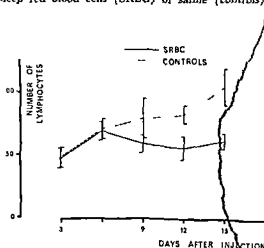


Fig 3 Number of lymphocytes with 0–5 mitochondria per mm³ of splenic artery blood at different intervals after a single injection of either sheep red blood cells (SRBC) or saline (controls) Mean ± S E

Veno Arterial Differences

The total number of lymphocytes per mm³ of splenic vein blood exceeded that in arterial blood in both immunized and control animals

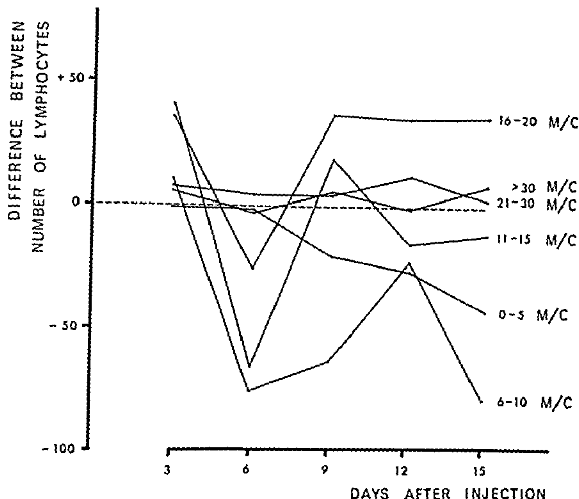
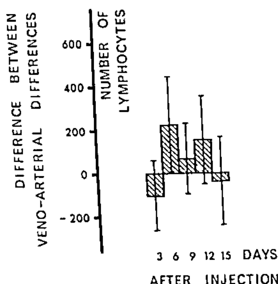


Fig 6 Mean difference between number of lymphocytes per mm³ of arterial blood in treated and control animals at different intervals after a single injection of sheep red blood cells (SRBC) and saline respectively The lymphocytes are subdivided into cells with a different number of mitochondria per cell (M/C)

number of lymphocytes with higher mitochondrial content (up to 20 mitochondria) (Fig 6)

Changes in circulating lymphocyte populations after antigen was studied earlier by *Ernstrom* & *Larsson* (1967) They reported a transient decrease in the percentage of low MC lymphocytes after two of the antigens used (sheep erythrocytes and *S typhi H*) At least after *S typhi H* antigen a low number of low MC lymphocytes in the peripheral blood was coincident with a low output of thymic lymphocytes indicating a possible relation between thymic function and peripheral blood lymphocyte content after antigen stimulation The decreased number of low MC lymphocytes following immunization with SRBC may also be due to increased consumption of these cells including a possible transformation into cells with higher mitochondrial content

There was a higher content of both low and medium MC lymphocytes in splenic vein blood than in arterial blood indicating migration of these cells from spleen to blood This splenic veno-arterial cell difference tended to decrease during the course of investigation as seen in the control animals The reason for this change in the control animals is not known It is probably not an effect of growth since in a number of untreated growing guinea pigs the splenic veno-arterial dif



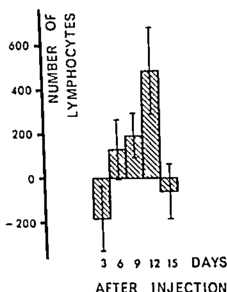


Fig 7 Change in splenic export of lymphocytes per mm³ of blood after a single injection of sheep red blood cells (SRBC) or pertussis vaccine Mean difference between injected animals and controls ± S E

ference in the number of lymphocytes in creases with age *(Sandberg* 1970)

In the animals treated with SRBC the slope in splenic veno arterial difference was interrupted by an increase in it on the 6th day after injection The values found on the 9th and 12th day were also higher than in the controls and this effect of SRBC was seen in both low and medium MC lympho cytes Therefore the reduction in the number of low MC lymphocytes in the blood ob served after SRBC was probably not due to a reduced migration of these cells from the spleen to the blood Total blood lymphocyte levels were unchanged in spite of increased splenic lymphocyte output

The haematocrit tended to be higher in splenic venous blood than in arterial blood The difference was too small (0–1 per cent) to appreciably influence the white cell counts

The effect of SRBC on lymphocyte migra tion from the spleen to the blood closely re sembles the effect of pertussis vaccine (studied by *Ernstrom* & *Sandberg* 1968) In both cases there was a decreased splenic veno arterial lymphocyte difference on the 3rd day after injection an increased difference on the 6th 9th and 12th day, and a decreased or normal difference on the 15th day (Fig 7)

Instead of being caused by an inhibition of the emigration the low splenic veno-arterial lymphocyte difference 3 days after SRBC and pertussis may be due to increased immigration of blood lymphocytes to the spleen This could represent a mobilization necessary for the coming increase in spleen cell prolifera tion and emigration It was previously shown that splenic response to antigen is dependent on the number of lymphocytes immigrating into the spleen rather than on the total num ber of lymphocytes in the spleen (*Ford* & *Gowans* 1967)

As regards splenic weight, hyperplasia of the spleen was reported to occur in mice *(Siefert* 1968) and rats (*Wissler et al* 1960 *Gunderson et al* 1962) with maxima on the 7th and the 4th day, respectively after SRBC or other particulate antigens In the present investigation splenic weight was slightly, but not significantly increased 6 and 12 days after SRBC

Thymic cervical and mesenterial lymph

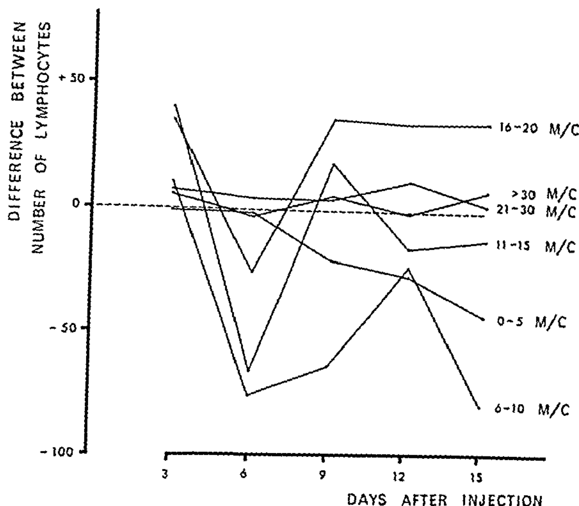


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ride at 8 C The height of the column was 110 cm and the diameter was 4 cm Thirteen ml of undialysed human serum were applied to the column The flow rate was about 25 ml per hour The effluent fluid was collected in fractions of 5 ml and the optical density of the fractions was measured at 280 mμ in a Beckman DB G spectrophotometer using a 1 cm cell The fractions were dialysed for 24 hours at 4 C against four changes of 0 85 per cent (w/v) saline solution

Starch block electrophoresis —Potato starch obtained from Jærens potetmelfabrikk Norway was washed several times in a buffer of pH 8 6 containing 0 012 M barbital and 5 5 ml 18 N sodium hydroxide per litre Small starch blocks 21 5 cm wide 29 cm long and 1 cm thick were used Serum was dialysed overnight against the barbital buffer and 12 ml of this serum were applied to the block Starch block electrophoresis was carried out at 4 C as described by *Kunkel* (1954) applying approximately 3 2 volts/cm Electrophoresis was carried out until the albumin had migrated about 15 cm from the origin The block was cut into section each 1 cm broad The proteins from each section were washed out with 10 ml of a 0 85 per cent (w/v) saline solution using suction The protein content of each fraction obtained was measured by the Folin method

Dialysis —Dialysis tubings from Arthur H Thomas Company Philadelphia U S A were used

Concentration of serum fractions —The concentrations were performed by dialysis against a saturated solution of Aquax (G T Gurr Ltd London) in saline at 4 C

EXPERIMENTS AND RESULTS

The Reaction between Serum E Y and Normal Human Sera

Normal human sera were tested for the Ag(x) antigen by means of serum E Y as well as with other anti Ag(x) sera The serum of one clinically healthy individual J V J scored as Ag(x—) from the negative reaction with six other available anti Ag(x) sera exhibited a precipitation reaction with serum E Y When serum J V J and a serum of type Ag(x+) were placed in neighbouring wells on an Ouchterlony slide and tested against serum E Y no reaction of identity developed between the two precipitates (Fig 1)

The precipitate between serum J V J and serum E Y was sharp and concave towards the antigen well located closer to the antigen well than the antibody well about ⅓ of the distance between them (Fig 1) The precipitate easily stained with the protein dye Thiazine Red In most experiments it did not stain with the lipid dye Oil Red O Occasionally, however a faint staining also with this dye was observed The lipid stain of this precipitate was however always much weaker than that observed in Ag(x) precipitates obtained with serum E Y

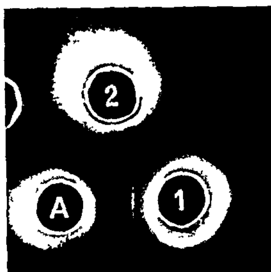


Fig 1 Test in agar gel showing the reactions between serum E Y and human sera The reagents were

Well A Antiserum (serum E Y) containing two precipitins

Well 1 Human serum of type Ag(x—) containing the E Y antigen (serum J V J)

Well 2 Normal human serum of type Ag(x+) lacking the E Y antigen

Note the lack of identity between the two precipitates obtained (in the native slide a reaction of non identity could be observed)

For convenience we will refer to the antigen demonstrable with serum E Y in the serum of the Ag(x—) individual (J V J) as the E Y antigen (to distinguish it from the Ag(x) antigen revealed by the same antiserum)

Identification of the Precipitating Component of Serum E Y

Serum of E Y was submitted to chromatography on a DEAE cellulose column to separate the γG globulin from the remaining

pooled four and four concentrated to about 2 ml and tested in Ouchterlony experiments The EY antigen was demonstrated only in the fractions from the first chromatographic peak No corresponding precipitates were observed after gel filtration of an Ag(x—) serum lacking the EY antigen

Immuno-electrophoresis was performed with 6 μl of a fraction possessing the EY antigen obtained from the gel filtration experiment After electrophoresis the longitudinal trough was filled with 200 μl of serum EY A precipitate in the α region developed after 2–3 days

Serum JVJ was submitted to starch block electrophoresis The eluates from each section of the block were concentrated to about 1 ml and tested in agar gel double diffusion experiments The antigen wells were filled twice Only the eluates from the β region formed precipitates with serum EY

The pooled concentrated fractions from the gel filtration experiment and the concentrated eluates from the starch block electrophoresis were tested against a number of specific rabbit anti human serum protein antisera in double diffusion tests using the small gel puncher No obvious correlation between the distribution of the EY antigen and any of the serum proteins was found

Frequency of the EY antigen

Serum samples from 222 healthy unrelated adult individuals from the Oslo area were tested against unabsorbed serum EY in double diffusion experiments In 92 of these serum samples precipitates were observed The sera were also tested against an anti Ag(x) serum (G D) which does not possess the EY antibody Among the 92 sera positive to serum EY 91 were of type Ag(x+) One of the serum samples JVJ was of type Ag(x—) but possessed the EY antigen This result was confirmed by testing this sample with absorbed serum EY Serum EY was absorbed with a serum of type Ag(x+) using the intra basin absorption technique This absorption removed the Ag(x) antibodies from the antiserum but not the antibody to the EY antigen The sera of type Ag(x+) were also retested with absorbed serum EY to test if any precipitate due to the EY antigen had been masked by the Ag(x) precipitates None of the 91 sera possessing the Ag(x) antigen reacted with the absorbed antiserum Thus among the 222 unrelated persons tested only one was found to possess the EY antigen (Table 1)

TABLE 1 *Distribution of 222 Norwegians with Respect to the EY and Ag(x) Antigens*

		No of individuals EY antigen +	—	Total
No of ind	Ag(x+)	0	91	91
	Ag(x—)	1	130	131
	Total	1	221	222

Preliminary Comparison between the SH and EY Antigens

Reference SH antigen and serum JVJ were tested in the same double diffusion experiment against both the SH Australia antibody of Prince and serum EY the latter absorbed in the well with a serum of type Ag(x+) Reactions of identity between the precipitates were demonstrated (Fig 5)

DISCUSSION

Serum EY was from a patient who had received multiple blood transfusions The two precipitins present in this serum are presumably antibodies formed in response to immunization with serum components not present in the serum of the patient This assumption is supported by the fact that both precipitins belong to the γG globulins Precipitating antibodies to components of human serum are found in some patients who have received multiple blood transfusions (*Berg & Bearn* 1966) The majority of these antibodies are ... is one of the antibodies in serum EY di... rected against serum β lipoprotein antigens

The reaction of non identity between the two precipitates formed with serum EY indicates that the two serum components react

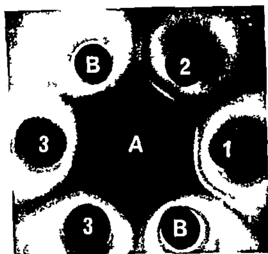


Fig 5 Double diffusion test in agar gel showing reactions of identity between the precipitates formed by the SH and E Y antigens and antibodies The reagents were

Well A SH Australia antibody (Sanders 4 × conc)
Well B Serum E Y absorbed with a normal human serum of type Ag(x+) lacking the E Y antigen
Well 1 *Human serum containing the E Y antigen* (serum J V J)
Well 2 Reference SH Australia antigen (CL 712 68)
Well 3 Normal human serum of type Ag(x+) lacking the E Y antigen

ing with this antiserum are antigenically different The distinction between these two antigens can be done safely only by employing absorbed serum E Y and proper controls In the following the antibody found in serum E Y *to a β lipoprotein antigen will not be* discussed further

The E Y antigen has thus far been only incompletely characterized The location and the shape of the precipitate indicated that the reacting component in serum J V J was of high molecular weight This notion was confirmed in the gel filtration experiment since the E Y antigen was excluded from the Sephadex G 200 gel

The staining properties of the E Y precipitate *indicate that a small amount of lipid* may be associated with the E Y antigen This view is compatible with the demonstration of a part of the E Y antigen in the fraction containing the high density serum lipoproteins from floatation experiments in the ultracentrifuge *However* since the greater part of the E Y antigen was found in the infranatant the employed method of centrifugation may have been inadequate for the purpose of separating completely the E Y antigen from the high density lipoproteins This problem obviously needs further study

None of the serum proteins traced by the immunological methods after gel filtration or starch block electrophoresis showed a distribution identical to that of the E Y antigen Therefore the E Y antigen was probably not part of any of the well known serum proteins tested for It appeared likely that the E Y antigen was part of a *hitherto unknown* serum component of high molecular weight

At the early stages of this investigation we had no reason to suspect that the E Y antigen was related to disease The antigen was observed in the serum of a blood donor J V J, in apparently perfect health and with no history of serious disease This fact suggested that the E Y antigen was not related to the so called Australia antigen which according to *Blumberg & Alter* (1965) and *Blumberg et al* (1967) is almost never found in healthy persons of Western European extraction

While the present report was *in preparation Prince* (1968a) reported the demonstration *of the SH antigen* which was strongly associated with serum hepatitis We have exchanged antisera with Dr Prince, and preliminary experiments conducted in both laboratories indicate that Dr Prince s and our antiserum may reveal the same antigen If extended immunological and association studies should provide decisive evidence that the two antigens are identical the preliminary notation adopted *for the antigen* revealed by serum E Y will be changed The nature of the association between the SH antigen and serum hepatitis is not established The antigen could be related directly to the infectious agent or represent a response to the infection (*Prince* 1968a) Preliminary studies by Prince (1968b) have suggested that the SH antigen and the Australia antigen are related

TABLE 1 *Presence of Hyperacute Rejection in 24 Presensitized Rabbits*

Code	Time before removal of the graft	Number of skin transplants	Preoperative cytotoxic titre	Acute interstitial allograft reaction	Hyperacute rejection pattern	Failure
223	1–5 hours	5	8			
235		4	8			
239*		9	0			
204		4	32			
208		4	2			
224	1 day	5	1		×	
240*		7	0	×		
217	2 days	4	16			
221		4	–			×
222		4	–		×	
229		9	8			×
230		7	1	×		
241		5	4		×	
218	3 days	3	0	×		
231		9	8		×	
232		9	1	×		
237		9	8	×		
238		7	1	×		
243		9	4		×	
244		7	2	×		
236	4 days	4	8		×	
225	5 days	5	2		×	
226		5	2			×
242		5	1			×

* In these two cases antibodies had been demonstrated earlier but were not demonstrable on the day of operation

Kidney Transplantation

Kidney transplantation was performed as an end-to-side anastomosis between the renal vessels of the donor kidney and the aorta and vena cava of the recipient (*Lund* 1969). Immediately after removal the donor kidney was perfused with 10 ml of cold saline in order to reduce the period of warm ischaemia. The temperature of the perfusion fluid was 3° C and the kidney was wrapped in cold saline-soaked gauze during the operation. The period of cold ischaemia averaged forty minutes (range 30–60 min.) including the time of perfusion.

After reestablishment of the circulation the graft was observed for colour changes and urine output for one to two hours before ureterovesical anastomosis was carried out.

Preparation

The kidney grafts were removed between one hour and five days after reestablishment of the circulation. Tissue for microscopy was fixed in 4 per cent aqueous formaldehyde and sections of paraffin-embedded material were stained with haematoxylin-eosin, phosphotungsic acid haematoxylin, Lendrum's stain for fibrin, periodic acid Schiff and sirius red for connective tissue.

RESULTS

The results are summarized in Table 1.

Cytotoxic Antibodies

Cytotoxic antibodies against donor leucocytes appeared in 21 of the 24 sensitized rabbits but were only demonstrated in 19 cases on the day of kidney transplantation.

The cytotoxic antibodies appeared about 14 days after the first skin transplantation and reached a maximum after six weeks.



Fig 1 Macroscopic appearance of the cortical necrosis in the kidney graft in case 225

Later skin transplants gave no further increase in the sensitization of the recipient

In none of the cases were cytotoxic antibodies observed before the first skin transplantation

Microscopic Examination

Evaluation of the microscopic sections was carried out as a blind test and three different patterns were observed

1 *Acute interstitial allograft reaction* which is mainly characterized by perivascular mononuclear cell infiltration This picture is analogous to that known from human allotransplantations

2 *Glomerular microthrombosis with cortical necrosis* Glomerular microthrombosis is defined as microthrombosis in more than ten percent of the glomeruli per section Microthrombosis in less than ten per cent of the glomeruli may also be found in autografts and is not uncommon in allografts from non sensitized rabbits Glomerular microthrombosis with cortical necrosis is characteristic for human hyperacute renal allograft rejection and this lesion will in the following be referred to as *hyperacute rejection pattern*

3 *Failures* characterized by cortical necrosis combined with thrombosis of the larger cortical vessels but without microthrombosis of the glomerular capillaries The percentage of such failures was the same in sensitized and non sensitized rabbits and since no other signs of rejection were found this pattern was ascribed to technical failure (*Lund 1970*)

Frequency of the Described Lesions

1 *Acute interstitial allograft reaction* In the immunized rabbits seven cases (of 24)

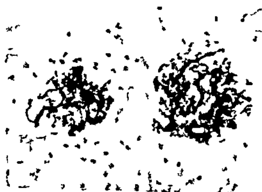


Fig 2 Glomerular microthrombosis and cortical necrosis in case 219 PTAH stain for fibrin

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ETHYLENE OXIDE RESISTANCE OF MICRO-ORGANISMS IN DUST COMPARED WITH THE RESISTANCE OF *BACILLUS SUBTILIS* SPORES

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(Head Ebbe Ahrensburg Christensen)

The resistance of standardized spore preparations of *Bacillus stearothermophilus* and *Bacillus subtilis* to industrial ethylene oxide sterilization procedures was compared Moreover the resistance of the most resistant preparation was compared with that of micro-organisms in dust and dirt The experiments demonstrated the importance of the water content to the ethylene oxide resistance of test pieces intended for the control of the microbiological effect of sterilization procedures It was demonstrated also that micro-organisms in dust and dirt may possess an ethylene oxide resistance in the same range as that possessed by *B subtilis* spores in the vacuum dried routine test pieces of Statens Seruminstitut Copenhagen Inactivation curves representing the subtilis strain and some of the micro-organisms which had survived the industrial sterilization procedures revealed that the decisive factor in the inactivation was the water content inside the micro-organisms or in their immediate vicinity

Most industrially produced disposable equipment for medical use is made partly or wholly, of plastic material which cannot tolerate sterilization by autoclaving or dry heat Such equipment has to be sterilized in the temperature range 20–80° C Sterilization methods available on an industrial scale are sterilization by ethylene oxide formaldehyde or irradiation

In sterilization by ethylene oxide the microbiological efficiency depends not only upon the concentration of ethylene oxide the time of exposure as well as the temperature but also to a marked extent upon the water content of the micro-organisms during the exposure (9 12 14) Physical measuring methods cannot record all of the important parameters At the present time therefore microbiological control is the only method which can disclose errors in the function of an ethylene oxide sterilizer with a reasonable margin of safety

As test organisms endospores are normally used either the spores of a subtilis variant *Bacillus globigii* (13) or spores of *Bacillus stearothermophilus* (3)

Very little is known about the relative resistance of the various micro-organisms to ethylene oxide (4 9 15) Accordingly it is not possible to relate the resistance of the named test organisms to the ethylene oxide

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resistance of the micro organisms commonly occurring in soil and dust

Since 1949 the Control Department at *Statens Seruminstitut Copenhagen*, has recommended a strain of *Bacillus subtilis* for use in the routine control of heat sterilization procedures in Danish hospitals This standardized spore preparation is still recommended for the control of dry heat formaldehyde and ethylene oxide sterilization (6)

In the present study the ethylene oxide resistance of the routine test pieces of Statens Seruminstitut will be related to the resistance of micro organisms in dust samples collected in a factory producing medical equipment

MATERIALS AND METHODS

The following microbiological preparations were used in the study

1 *Bacillus subtilis* spore samples of Statens Seruminstitut for controlling sterilization by dry heat formalin and ethylene oxide
2 *Bacillus stearothermophilus* spore samples of Statens Seruminstitut for controlling autoclaving procedures
3 Dust samples
4 Strains of bacilli isolated from dust treated with ethylene oxide in that part of the experiments which was run as industrially employed sterilization procedures (*vide infra*)

Each of the Serum Institute samples of subtilis spores consists of 2 × 10^6 unwashed spores vacuum dried in sand For making spore sand plate culture from 50 agar plates (diameter 14 cm) incubated for 5 days at 37 C was used The total quantity of plate culture was suspended in physiological saline and then mixed with sterilized quartz sand About 3 ml of physiological saline as used per gramme plate culture and approx 1000 g of sand (E Merck AG Germany) was used for absorption of 200 ml spore suspension Immediately after the mixture the preparation was vacuum dried at a pressure of 2–4 mm Hg and finally homogenized in a mortar Drying period 24 hours The spore sand in the form of test pieces was used in quantities of 120 mg wrapped in two layers of paper

The same technique was used for preparing pore sand of some of the strains of bacilli isolated in the course of the study from dust treated with ethylene oxide In these cases however culture from only 10 agar plates was used

The stearothermophilus test pieces were prepared and standardized by Dr *I Juhlin* Bacteriological Institute of the Malmo General Hospital Sweden The test pieces consisted of two filter discs packed in paper each containing approx 10^7 spores The preparation had been dried in a freeze drying plant (10) These spore samples are used in Sweden Norway and Denmark in the control of autoclaving procedures

The dust samples were derived from 8 air filters collected in manufacturing halls for disposable medical equipment The dust was homogenized by sifting and used as samples containing 10^4 or 10^6 viable units During the study period the dust samples were stored at a relative humidity ranging from 45 to 60 per cent

In the first second and third experimental series the ethylene oxide treatments were performed under industrial conditions The sterilization procedures were carried out in a factory producing medical equipment* The methods were the routine sterilization procedures at 55 and 75 C used by the factory Saturated water vapour was used for heating the utensils to be sterilized In both programmes the effective treatment period was approx 5 hours and the ethylene oxide concentration in the sterilization chamber was about 1100 mg/litre gas phase The sterilizers had been designed by the factory engineers

The ethylene oxide exposures during the fourth experimental series were carried out in the Control Department Statens Seruminstitut using an old autoclave which had been rebuilt so as to heat the sterilization chamber through a thermostat regulated water casing adjusting the temperature with an accuracy of ± 1 C The pressure in the sterilizing chamber could be read with an accuracy of ± 2 mm Hg The same procedure was used for all the exposures in the experimental oven The sterilization chamber was evacuated to a residual pressure of 20 mm Hg and water was supplied to the chamber making the relative humidity 60 per cent An ethylene oxide freon mixture containing 12 volume per cent ethylene oxide was added to obtain a partial pressure corresponding to an ethylene oxide concentration of 200 mg/litre The pressure was adjusted to atmospheric by adding nitrogen The exposure period was taken to be from the time that the supply of ethylene oxide freon gas was discontinued until a final evacuation was instituted In all cases the temperature was 35 C

Preparations which had been exposed to industrial sterilization procedures were cultured in liquid media The specimens of subtilis spores and the dust specimens were cultured in Beef broth

* The author is indebted to Mr *Lars Berit* civil engineer managing director of Sterntex Ltd Fspergærde Denmark for his helpfulness

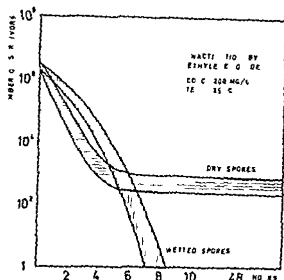


Fig 1 Inactivation curves for *B subtilis* spore test pieces of Statens Seruminstitut partly vacuum dried for 24 hours (8 mm Hg) and partly stored for 24 hours in saturated water vapour prior to ethylene oxide exposure

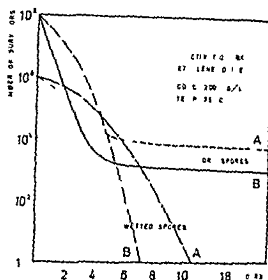


Fig 2 Inactivation curves for spores of two strains A and B isolated from dust samples treated with ethylene oxide. The micro-organisms are classified as *Bacillus* strains. The preparations of spores had been partly vacuum dried for 24 hours (8 mm Hg) and partly stored in saturated water vapour for 24 hours prior to the ethylene oxide exposure

of fungi were isolated from the dust samples treated with ethylene oxide

In the *fourth experimental series* inactivation curves were determined for the subtilis test strain and for some of the micro-organisms which had survived the ethylene oxide treatments in the preceding experimental series. Fig 1 illustrates the inactivation curves for wetted and dry subtilis spores. A number of subtilis test pieces had been stored in saturated water vapour at room temperature for 24 hours prior to the ethylene oxide exposure while others were vacuum dried as customary with test pieces. The experiment showed that spore specimens adequately wetted prior to the treatment in the experimental oven were inactivated approximately exponentially with the exposure period while the inactivation curve for dry subtilis spores was not a straight line. About 0.1 per cent of the initial spore count were micro-organisms having a greatly increased resistance.

Corresponding inactivation curves for spores of two of the isolated strains of bacilli are shown in Fig 2. The spore preparations of these strains were produced in the same way as the preparation with subtilis spores and were treated like the latter prior to the treatment with ethylene oxide. Both of the newly isolated strains showed inactivation curves of the same shape that was found for the subtilis strain. In both cases part of the spores in the preparation which had not been wetted prior to the ethylene oxide treatment were extremely resistant to ethylene oxide. In strain A about 1 in 10 and in strain B 1 in 10³ of the initial spore count was almost entirely unaffected by the ethylene oxide treatment

DISCUSSION

Medical equipment to be sterilized by ethylene oxide comprises not only industrially manufactured disposable equipment such as infusion sets, injection syringes, needles, catheters etc but also expensive and often complicated equipment such as cystoscopes, other endoscopes, parts of anaesthetic apparatus etc. It applies to both groups that the contamination which is to be inactivated by sterilization usually consists of micro-orga

nisms from airborne dust and from human skin and mucous membranes Some of the micro organisms found on the material, there fore have become desiccated in environ ments containing organic material and vari ous inorganic salts e g sodium chloride

Abbott et al (1) have demonstrated that spores can survive gas sterilization if they are encapsulated in crystalline material *Beeby & Whitehouse* (2) have reported moreover that samples of *B globigii* spores suspended in physiological saline prior to desiccation on aluminium foil—as the only ones of five ex perimental preparations of this micro orga nism produced by different suspension media —could not be inactivated in their experi mental oven or in four commercially manu factured ethylene oxide sterilizers when used in the recommended standard programmes *Phillips* and his co workers in 1961 (12) and 1964 (9) demonstrated that strains such as *B globigii* and *Staphylococcus aureus* many acquire a greatly increased resistance if they are exposed to a relative humidity below 30 per cent and that a very long time is required to rehydrate micro organisms thus desiccated That rehydration of dry micro organisms prior to ethylene oxide exposure may be a presupposition of inactivation has often been demonstrated (5 13 16)

From other sterilization methods we know examples of micro organisms which have an extremely high resistance to the method con cerned For instance compared with the pathogenic spore forming bacteria spores of *B stearotheromphilus* (11) have a very high resistance to autoclaving and compared with the most radioresistant Bacillus and Clostri dium strains the *Micrococcus radiodurans* (7) possesses an extremely high radioresis tance So even though it is possible in the named examples to alter the resistance greatly by modifying the technique of prepa ration the strains do possess a special prop erty By modifying the technique of prepara tion it is not possible to lend subtilis spores the same resistance to autoclaving as that found in spores of *B stearothermophilus* in a preparation of medium quality and it is not possible without the use of very special artificial means to render spores of *Bacillus pumilus* as radioresistant as *M radiodurans* is in a conventional preparation The resist ance to ethylene oxide acquired by a bacterial strain on drying under conditions where crys tals are formed appears to depend primarily upon physical phenomena The common place pathogenic micro-organisms too may acquire this high resistance if they can toler ate the dehydration Therefore this pheno menon is of decisive importance in the use of ethylene oxide for sterilization purposes, and a micro biological control of ethylene oxide sterilization should be planned with a view to disclosing sterilization procedures which have not effectively rehydrated the micro orga nisms and disorganized watersoluble crystals

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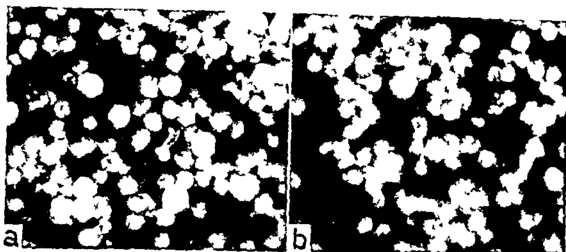


Fig 1 EB-3 cells treated with an EBV antibody-containing serum (a) and with a negative serum (b)

period of 15 years were available from 4 persons without a history of IM A positive reference serum was kindly supplied by Dr Werner Henle Philadelphia USA and a selected human serum which consistently appeared negative in the immunofluorescence test was used as a negative reference

Cells The EB 3 cell line kindly supplied by Dr Werner Henle was used throughout the study The cells were grown in Erlenmeyer flasks and incubated at 37 C using a 100 ml bottle for 20 ml of cell suspension

Media The cells were maintained on Minimum Essential Medium Eagle with 10 per cent Foetal Calf Serum (Microbiological Associates Inc Bethesda Md) 0 08 per cent $NaHCO_3$ and 200 i u penicillin and 0 05 mg streptomycin pr ml This medium was changed every 3 to 4 days Late in the study however the medium was changed only once a week in order to enhance the EBV infection (10) Before the cells were used in the immunofluorescence test they were kept on Eagles Basal Medium with 25 per cent Foetal Calf Serum for 5 to 7 days (11)

Preparation of cell smears The cells were centrifuged at 900 r p m for 10 minutes and after removal of the medium they were washed twice in Phosphate Buffered Saline pH 7 4 (PBS) After resuspension in a small amount of PBS approximately 2 × 10 viable cells were placed on microscope slides air dried at 37 C and fixed in acetone at room temperature for 10 minutes The slides were stored at minus 20 C until used

Staining Serum dilutions were applied to the dried and fixed cell smears and incubated for one hour in a moist chamber at 37 C After washings in PBS the smears were overlaid with goat anti human IgG conjugate (Hyland Laboratories, Los Angeles California) and then incubated and washed as described above After a brief rinsing in destilled water and mounting in PBS and glycerol (4 1) the slides were examined in a Zeiss microscope with an Osram HBO 200 lamp

Titration of antibodies Titrations were performed employing twofold dilutions of sera in PBS beginning with 1 10 In smears exposed to strongly positive sera in low dilutions the percentage of fluorescent cells varied between 5 and 15 The number of fluorescent cells as well as intensity of the staining decreased gradually with increasing serum dilution and corresponding with this the reactions were given the scores 3 2 1 and 0 Fig 1 shows a positive field given the score 3 and a negative field given the score 0 respectively The serum titre has been expressed as the reciprocal of the highest dilution given the score 2 The titration of all sera from one patient was performed simultaneously in one experiment on smears from the same batch and all slides were randomized and read blind Positive and negative reference sera were always included in the assay In 45 titrations of the positive reference serum the titre has been 160 twice 320 on 28 occasions and 640 15 times

Paul Bunnell test The Paul Bunnell reaction a.m. Davidsohn was kindly carried out by Dr E Kjems at the Streptococcal Department Statens Seruminstitut

RESULTS

The results of the EBV antibody determinations by the indirect immunofluorescence method on serial sera from 33 patients are recorded in Table I It will be seen that the first of the patients listed No 101 shows a traditional antibody curve in that a 4 fold

TABLE 1 *EBV Antibody Titres in 33 Patients with a Clinical Diagnosis of Infectious Mononucleosis*

Pt no	Week after onset of disease 1	2	3	4	5	6	7	8	9–12	13–16
101		40	160	160	80	80	40	10		
102	640	320	160		320	320			160	
103		80	20	40	20				40	
104	160	160	80	40	80				40	80
107			320	320	320	320	320		320	160
108		640	640		640			160		160
109		160	160	80		160			160	160
112				320	320	160	80		80	
113	160	160		40		80			40	80
114		640	320	160	160	160			160	
115				80	80	40	40		20	
116						160	160	160	160	320
119					160		160		80	160
120	40	40	20						320	320
121			640	640		320			160	160
122					640	1280	320	80	160	160
124		640	640	160	320	320			160	
126	160	160		160				160		
127		320		320	80				40	
128	2560	640		320					320	
129	320	320					160		320	
131		160	160	160				40		
132			320	160	160				160	
134			320	160	160	160				
136			80	40	40	40	40		40	
139		320	160	320	160				320	
140				80	80	160			160	
141		640	320	160	160				160	
142			320	2560	1280	320				
145		320	640	160	40	40	80			
146		80	80	40	40					
151	80	80	40	40	80					
153		80	40	20	20					
Geometric mean titres	226	201	166	133	131	160	106	77	130	160
No of sera in group	8	21	22	26	24	17	10	6	23	10

rise in antibody titre from 40 to 160 occurs between the third and eighth week. Patient No 142 shows an eightfold rise in titre from the third to the fourth week while patient No 120 in the ninth week has a titre four fold higher than that in the third week after onset.

However such antibody changes are not representative for the patients in the present study in that the majority 18 individuals show a significant (four fold or more) decrease in antibody titre during the study period. In the remaining 12 patients constant titres were found throughout the observation period. It should be mentioned that for patient No 151 a serum specimen collected 7 months before onset of illness was available and found to be free of EBV antibodies.

In the control group 27 sera showed titres ranging from 10 to 640 while the remaining 8 persons did not have antibodies. The maximum EBV antibody titres ranged from 80 to 2560 for the IM patients.

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ISOLATION OF LYMPHOCYTES FROM BLOOD

A Procedure Combining Nylon Fibre Filtration and Differential Centrifugation

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Lymphocytes were isolated from human blood by a simple procedure combining nylon fibre filtration and centrifugation in colloidal silica polyvinylpyrrolidone In the preparation obtained 99 5 ± 0 6 per cent of the leukocytes were lymphocytes it contained 0–202 mostly less than 20 platelets and 0–1 5 mostly 0 RBC per 100 leukocytes The lymphocyte recovery was 13 3 ± 3 3 per cent In the trypan blue test 2 2 ± 3 0 per cent of the cells were stained

Two main principles have been employed in the separation of human peripheral lymphocytes adhesion of cells to surfaces and centrifugation in media of different density (For reviews see *Ling* 1968 and *Cartier et al* 1969)

Selective adhesion of blood cells to fibrous materials first employed for lymphocyte isolation by *Fichtelius* (1951) separates other leukocytes and platelets but not red cells from the lymphocytes (*Rabinowitz* 1964)

Centrifugation in density gradients well separates polymorphonuclear (PMN) and red blood cells (RBC) from the lymphocytes but for platelets and monocytes various results have been obtained (*Ling et al* 1965 *Noble & Cutts* 1968 *Pertoft et al* 1968)

The purpose of this investigation was to develop a simple procedure for the isolation of human peripheral lymphocytes A combination of the selective adhesion and centrifugation techniques was used

Received 21 x 69

MATERIALS

Materials were obtained from the following sources Heparin from Vitrum AB Stockholm Sweden sterile disposable nylon fibre filters LeukopaK® from Fenwal laboratories Morton Grove Illinois glutathione and cover glass cement according to Kroenig were obtained from Merck AG Darmstadt Germany Parker TCM 199 from Flow laboratories Irvine Ayrshire Scotland a colloidal silica solution Ludox® HS (40 per cent w/w in deionised water density 1 295) from du Pont de Nemours Wilmington Delaware polyvinylpyrrolidone mol wt 4 × 10⁴ from Thomas Co Philadelphia Pennsylvania a modified Eagle s solution Isoton and Zaponin® from Coulter Electronics Ltd Dunstable England

METHODS

Obtaining Samples of Blood

Venous blood was obtained from healthy donors Each sample of 400 ml was collected into a sterile bottle containing 6000 IU of heparin in 4 ml saline and divided into two parts 100 and 300 ml respectively The separation was started within an hour of bleeding and in the meantime the blood was handled at room temperature

TABLE 2 *The Presence of RBC and Platelets in Lymphocyte Preparations*

Run no	Filtered blood	Purified lymphocyte suspension	
	Platelets per 100 leukocytes	Platelets per 100 leukocytes	RBC per 100 leukocytes
1	352 0	201 5	0
2	20 3	168 7	0
3	3 2	13 0	0 75
4	2 0	0 25	0
5	4 6	2 2	0
6	4 2	2 2	0
7	2 7	1 ~	0
8	49 0	16 1	1 5
9	0 7	1 0	0
10	8 3	0 75	0
11	29 6	13 7	0
12	9 6	5 0	0
13	118 7	33 ?	0
14	21 7	3 5	0

TABLE 3 *The Recovery of Lymphocytes Per Cent of Cells in the Original Blood Sample**

Filtered blood	Non filtered centrifuged cell suspension	Purified lymphocyte suspension
74 1±18 4	30 7±7 2	13 3±3 3

* *Mean ± 1 S D of 14 runs*

1) RBC occurred in about the same number as the leukocytes

Combined Filtration—Centrifugation

In the MN band 99 5 ± 0 6 per cent of the leukocytes were lymphocytes 0 2 ± 0 2 per cent PMN leukocytes and 0 3 ± 0 6 per cent monocytes (Table 1) There were 0–1 5 mostly 0 RBC and 0–202 mostly fewer than 20 platelets per 100 leukocytes (Table 2) The recovery of lymphocytes from the original blood sample was 13 3 ± 3 3 per cent (Table 3) The purified lymphocyte suspension is illustrated in Fig 2b

Viability Test

In the trypan blue test buffy coat cells investigated before as well as after filtration all excluded the dye Of the non filtered cells centrifuged in density cushions 0 7 ± 1 1 per cent were stained in the MN band and 1 3 ± 3 0 per cent in the PMN band In the purified lymphocyte suspension 2 2 ± 3 0 per cent of the cells were stained

DISCUSSION

The procedure described is relatively simple and permits the separation of highly purified lymphocytes The viability of the separated cells is well preserved and the recovery is satisfactory The final lymphocyte suspension is usually obtained within 5 hours of bleeding thus experiments with the lymphocytes can be started in the afternoon of the same day

In the first step lymphocytes and RBC are separated from PMN cells monocytes and platelets due to differences in adhesiveness to nylon fibres In the second step based on differences in density the lymphocytes are separated from RBC and the number of PMN cells is further reduced

In column separation irrespective of the materials used, more than 95 lymphocytes per 100 leukocytes are usually obtained (*Lang* 1968) In the experiments involving adhesions to glass wool and glass beads *Rabinowitz* (1964) obtained 1 PMN leukocyte 0 monocyte and 18 platelets per 100 separated lymphocytes But, as in most techniques depending on sedimentation in a rouleaux forming agent for RBC removal there were more RBC than lymphocytes in the final suspension Apart from the RBC the purity of the lymphocyte preparations obtained by *Rabinowitz* was about the same as that reported in the present study

Techniques involving separation in density gradients are generally more complicated than the present one which when only lymphocytes are to be isolated requires preparation of a single density cushion *Noble & Cutts* (1968) centrifuging blood in ficoll gradients obtained lymphocyte preparations with few PMN cells RBC and platelets Their technique in contrast to the use of *Lang* (1965) using albumin and *Pertoft et al* (1968) using colloidal silica and polyvinyl

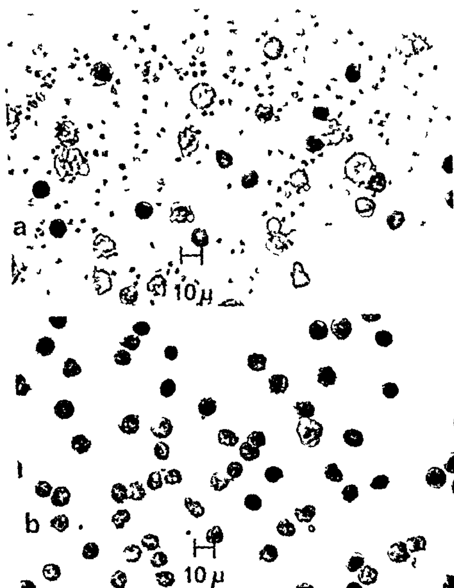


Fig 2 Separation products a) Non filtered centrifuged cell suspension MN band b) Purified lymphocyte suspension

pyrrolidone had the remarkable ability to completely separate the lymphocytes from monocytes *Perper et al* (1968) combining glass bead filtration with a centrifugation procedure obtained lymphocyte preparations free even from platelets but compared to the present technique the preparation of centrifugation media was very elaborate

Among factors important in lymphocyte isolation *i e* purity recovery viability and technical simplicity viability has been only partly studied in this investigation the trypan blue test being comparatively rough (*Rabinowitz* 1964) Culture experiments in progress will give more information about viability

This investigation was supported by *the Swedish Cancer Society and by the Faculty of Medicine University of Umeå* I am grateful to Dr *Bertil Cedergren* who kindly provided blood samples.

cytoplasm of HeLa (25) and KB (10) cells were reported they were not observed in other systems Since different strains of VSV were used in most of these investigations and the conditions for infection were variable it is difficult to establish a uniform concept as to how in the final stage of replication this virus leaves the infected cells With the exception of one study (4) which claims the *presence of unusual intracellular* structures associated with the multiplication of VSV in chick embryo fibroblasts close scrutiny by others disclosed no such cellular deposits Morphologically well differentiated particles either at the cellular periphery or in cytoplasmic vacuoles constituted the only evidence of infection

Staining with fluorescent anti VSV rabbit gamma globulin has been extensively in use for many years to demonstrate and count infected L cells (11) as well as for assay of antiserum against L cell interferon (2) The uniform cytoplasmic distribution of antigenic foci in L cells within hours after infection suggested that the cytoplasm of infected cells may contain deposits of non differentiated viral protein which might be amenable to serologic identification in the electron microscope An opportunity presented itself therefore to determine whether there existed a correlation between the fluorescent patches of infected cells seen in the light microscope with any areas within the cytoplasm which may bind ferritin conjugated antibody and which can be visualized in ultrathin sections by electron microscopy

MATERIALS AND METHODS

Viruses The New Jersey type of VSV was propagated in chick embryo fibroblasts as described elsewhere (6)

Cells The MCN line of L cells (1 cultivated in Scherer s maintenance solution (60 per cent medium 199 (30 per cent) and horse serum (10 per cent) supplemented with sodium bicarbonate (0 26 per cent) and antibiotics was used throughout HeLa cells originally derived from the S3 clone (12) were grown in Sella s medium with the addition of glucose (5 per cent) horse serum (10 per cent) antibiotics and sodium bicarbonate (0 13 per cent)

Immune sera Antisera were prepared in rabbits by a series of intravenous inoculations. Neutralizing titres as determined by inhibition of approximately 100 plaque forming units (p f u) of virus ranged from 10 [illegible] to 10 [illegible] per 0 2 ml

Conjugation of antibody with fluorescein Anti VSV sera were subjected to conjugation with fluorescein isothiocyanate (Sylvana Chemical Corporation Millburn N J USA) according to the original method of Coons (3) as modified by *Spendlove* (24) Neutralizing titres of fluorescein linked antibody determined by inhibition of VSV plaques were in excess of 10 [illegible] per 0 2 ml of serum

Conjugation of antibody with ferritin Both the m xylene and toluene diisocyanate methods (21 22) as modified by *Rifkind et al* (15) were used. The final products were subjected to immune electrophoresis according to *Borek & Silverstein* (1) Conjugation was deemed successful when full coincidence was achieved between the immune precipitates of antibody conjugate and rabbit anti ferritin and sheep antirabbit gamma globulin sera respectively Successful conjugates possessed neutralizing titres in an order of magnitude of 10 [illegible] to 10 [illegible] per 0 2 ml

Infection of cell cultures Roux bottles containing about 3 × 10[7] HeLa cells or about 6 × 10[7] L cells were drained and inoculated with 1 ml of virus suspension For infection at high dilution an input multiplicity (m) of 0 01 was used When a high virus cell ratio was desired m was approximately 10 Adsorption was permitted to proceed for 1 hour at 37 C and the bottles were rocked occasionally to spread the inoculum evenly over the surface of the monolayer The cells were then washed twice with 50 ml of Hanks solution refed with the appropriate medium and returned to the incubator Media or cells were harvested at the times indicated in the text Samples of media were stored in ampoules at − [illegible]0 C until infectivity assays could be performed Cells were harvested by scraping into 4 5 ml of the corresponding growth medium or of 0 1 M phosphate buffered saline (PBS and centrifuged at [illegible]00 g for 5 minutes The pellets were resuspended in 5 ml of 0 1 M sodium cacodylate buffer containing 3 per cent glutaraldehyde unless otherwise mentioned Prefixation was allowed to proceed for 10 minutes at room temperature The tubes were then centrifuged once again for 10 minutes at [illegible]00 × g the fixative was poured out and the dense pellets were prepared for electron microscopy as described in a following paragraph

Tagging of cells with ferritin antibody conjugate Pellets of cells prefixed in glutaraldehyde as described in the preceding paragraph received each 2 drops of 0 88 M sucrose and were then frozen and thawed once in a dry ice alcohol bath. This step was later shown to be detrimental to

preservation of cellular integrity and was omitted in some experiments 0 2 ml of anti VSV ferritin conjugated serum were then added and the mixture was incubated for 1 hour at room temperature with intermittent shaking The cells were washed twice in PBS using 20 ml for each centrifugation in order to remove excess antibody and ferritin The final pellet was then prepared for electron microscopy

Modification of the Ferritin Technique

a *Cryostat sections* L cells in various stages of infection with VSV were scraped into a small amount of regular growth medium and spun at 200 × g for 5 minutes The pellets were resuspended in 0 2 ml of 0 88 M sucrose transferred to ampoules and quickly frozen in dry ice alcohol For cutting in a freezing cryostat (Bright s Ltd London England[1]) the frozen cell suspension was dislodged from the glass by gentle thawing and immediately transferred to the cryostat holder for sectioning at —20 C The individual sections had a thickness of approximately 4 μ They were collected in a watch glass permitted to thaw and without prefixation in glutaraldehyde were incubated for 1 hour at 37 C in the presence of 0 5 ml of undiluted ferritin conjugate Further handling was already described

b *Treatment with digitonin* Pellets of infected cells were suspended in 4 × 10 M digitonin and incubated for 5 minutes at room temperature This step was followed by two centrifugations in PBS and the cells were subsequently prefixed in glutaraldehyde and incubated with ferritin antibody as mentioned above

c *T atment with dim thylsulphoxide (DMSO)* Following prefixation in glutaraldehyde as already outlined cells were washed once in PBS by centrifugation and resuspended in 10 per cent DMSO in 0 1 M PBS at pH 7 4 Incubation was carried out for 10 minutes at room temperature while the cells were kept in suspension on a magnetic stirrer After two centrifugations in PBS they were incubated with ferritin antibody and processed for electron microscopy in the usual manner

d *Chopping of cells* After routine fixation in 3 per cent glutaraldehyde cell pellets were enrobed in 5 per cent Nobel agar prepared in 0 1 M PBS at pH 7 4 to which 4 5 per cent sucrose had been added The agar blocks were chopped in a tissue sectioner (Ivan Sorvall Inc Norwalk Connecticut USA) (23 nd the ma h was stored overnight in PBS containing 0 2 M sucrose

This procedure was followed by two centrifugations in the same buffer exposure of the cell agar sediment to ferritin antibody and the materials were further processed as usual

Preparation for electron microscopy Pellets of cells which had undergone prefixation in glutaraldehyde followed or not by incubation in the presence of ferritin antibody were enrobed in 1 5 per cent Nobel agar made up in Michaelis buffer at pH 7 3 with the addition of 4.5 per cent sucrose The agar was kept in a waterbath at 45 C and the cells suspended in this manner were spread thinly on a glass slide Upon solidification the agar was cut into blocks approximately 1 mm³ in dimension These were transferred for 1 hour at room temperature into 1 per cent OsO_4 in Michaelis buffer pH 7 3 containing 1 5 per cent sucrose Thereafter the blocks were washed twice in the same buffer and dehydrated in graded dilutions of ethanol After treatment with propylene oxide (9) the blocks were finally embedded in Vestopal W (Jaeger Vesenaz Geneva Switzerland) (18) Sections approximately 300 500 A in thickness were cut with a microtome (LKB Ultratome III) collected on carbon reinforced Formvar coated 200 mesh copper grids and examined in the electron microscope (Philips EM 100) after staining with magnesium uranyl acetate (5) and lead citrate (11) Exposures were made at primary magnifications of 1 400 and 8 700 on Kodak Fine Grain Safety Positive 35 mm film and prints were obtained after photographic enlargement as le sired

Fluorescent microscopy Coverslip tubes containing approximately 2 × 10 cells of either type were infected with 0 2 ml of virus dilution at the required input multiplicity of ... 10 At ... vals coverslips were withdrawn washed gently several times in PBS and permitted to dry at room temperature before fixation for 10 minutes in acetone Subsequently they were overlayed with fluorescein conjugated anti VSV gamma gl b ulin and incubated for 1 hour at 37 C The covers slips were then washed for 5 minutes each time in three changes of PBS and three changes of distilled water in st ... vessels ... on a magnetic stirring device After drying at room temperature they were mounted with Elvanol (16) on glass slides and examined in the fluorescent microscope

Infectivity assays Titrations were performed in 30 ml plastic vessels (Falcon Plastics Corp Los Angeles Calif USA) containing 3 × 10⁵ cells of either type Before infection they were drained and inoculated with 0 1 ml ... units of ... fold dilutions of virus in Hanks solution Incubation proceeded for 1 hour at 37 C with intermittent rocking of the bottles They were subsequently overlaid with ... ice the regul r ... ncentration ...

Kindly made available by Dr Figgo Fab Autoimmune Laboratory Statens Seruminstitut Copenhagen Denmark

Kindly made available by Dr H Moe and J Rostgaard Anatomy Department C University of Copenhagen Denmark

an equal volume of 2.4 per cent agar. Neutral red 1:30 000 was included in the overlay for L cells but not for HeLa cells. Upon completion of 48 hours of incubation the latter received a second overlay containing neutral red diluted 1:8 000. Plaques were counted on the third day and titres were expressed as p.f.u. per 10 cells.

RESULTS

Multiplication of VSV in L and HeLa Cells

a. *Development of infectious virus.* The growth patterns of VSV in L and HeLa cells under conditions of overwhelming infection (m = 10) are illustrated in Fig. 1. Small increases in infectivity titre above residual levels were measurable 4 hours after infection, although earlier time intervals were not tested. Nearly maximal amounts of infectious virus were liberated from both types of cells by 8 to 10 hours. Final titres in HeLa cells were approximately 10 times higher than those obtained in L cells.

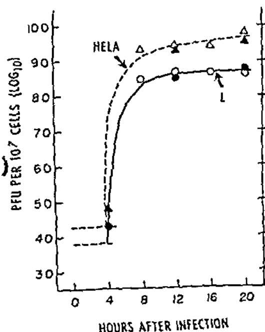


Fig. 1. Multiplication of VSV in L and HeLa cells. Open and closed symbols represent different experiments.

b. *Development of viral antigen.* The application of anti-VSV fluorescent gamma globulin to smears of L and HeLa cells infected for varying lengths of time with VSV (m = 10) revealed changes illustrated in Fig. 2. Uninfected L cells (Fig. 2a) were devoid of any fluorescent deposits. Within 6 hours after infection many cells exhibited numerous patches of viral antigen distributed throughout the cytoplasm. Size of the antigenic deposits was variable and they were absent in the nucleus (Figs. 2b, 2d). At 13 hours post infection, the cytoplasm of many cells had fused into multinucleated structures (Figs. 2c, 2e). Antigen remained restricted to the cytoplasm and no fluorescence was discernible in the nuclear region. A few hours later the culture was all but destroyed.

In HeLa cells exposed to VSV damage appeared to progress even more rapidly. Normal cells are depicted in Fig. 2f. Six hours after infection with a large dose of virus the cultures displayed extensive clumping. Distribution of fluorescence was more diffuse and often not as clearly defined as in L cells (Fig. 2g). At 12 hours post infection the cultures had degenerated further (Fig. 2h). Syncytial forms were absent.

Problems Encountered in the Use of Ferritin Conjugated Antibodies for Staining of Intracellular Virus

Distinct virus particles were often recognized in vacuoles of cells infected 18 hours earlier with dilute inocula of VSV. These particles which presented all the morphological attributes generally associated with extracellular virus constituted the only intracellular evidence of infection. The procedure for introducing ferritin linked antibody into the cell was therefore checked out with cells infected in the above manner.

In first attempts L and HeLa cells which had been infected 18 hours previously with VSV at a multiplicity of 0.01 were prefixed in 5 per cent formalin, frozen and thawed and exposed to the ferritin conjugate as previ

ously described The samples were then handled further as outlined in Materials and Methods

Examination of these materials in the electron microscope revealed a profound disorganization of intracellular detail which made it difficult to decide whether the tagged virus had originated from the interior or from the surface of the cell Glutaraldehyde in concentrations ranging from 3 to 0 75 per cent was therefore substituted for formalin Fig 3 shows a portion of a HeLa cell infected 18 hours earlier with VSV which was subjected to prefixation in 3 per cent glutaraldehyde This method of fixation was found considerably milder than formalin Moreover antigenicity of the preparation had remained intact as shown by the association of the ferritin label with extracellular virus structures However the antibody did not appear to have gained entry into the cell There was no background staining and morphologically identifiable virus located in intracytoplasmic vacuoles was not tagged (not shown in the figure) Noteworthy were also vacuoles filled with what appeared to be hollow forms of virus which exhibited cores of low electron density (Fig 4) Not infrequently HeLa cells infected with a low multiplicity of VSV (0 01) displayed characteristic whorls of endoplasmic reticulum as illustrated in Fig 6 At a higher magnification it can be recognized that the cisternae of the reticulum are lined with an evenly spaced succession of ribosomes (Fig 5) However such arrangements of the endoplasmic reticulum were sometimes observed as well in uninfected HeLa cultures maintained under suboptimal conditions Therefore it is likely that the whorls are a reflection of impaired metabolic function and may not represent a specific change associated with viral replication

It proved impossible to attach ferritin antibody in any specific manner to virus structures in intracytoplasmic vacuoles Hence it was also considered unlikely that non differentiated deposits of viral antigen could be pin pointed by means of ferritin tagged antibody More drastic attempts at opening cells without destroying intracellular detail were therefore considered These included

a) Sectioning of frozen non fixed cell pellets in a cryostat to a thickness of approximately 4 µm followed by incubation with antibody fixation, etc. in the usual manner

b) Pretreatment of cells with digitonin

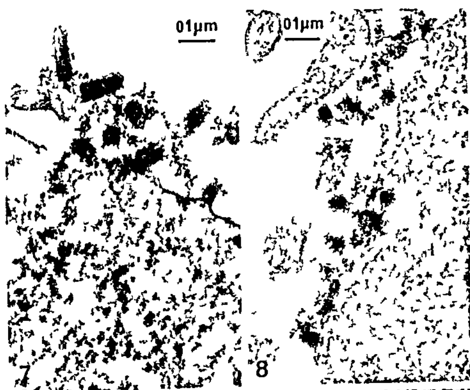
c) Pretreatment of cells with dimethyl sulphoxide (DMSO)

d) Chopping of non fixed cells embedded in agar by means of a tissue sectioner

The results of these efforts were uniformly negative In no instance was it possible to associate ferritin antibody with anything but well differentiated virus at the cellular surface Only when cells approached a state of total dissolution or had been opened mechanically did ferritin antibody find its way into the cell There it appeared to attach indiscriminately to non definable fibrillar structures A HeLa cell in this condition 21 hours after infection with VSV is shown in Fig 7 Both tagging of extracellular or membrane attached virus and distribution of ferritin throughout the cytoplasm can be seen However uninfected HeLa cells about to disintegrate following repeated cycles of freezing and thawing presented a similar appearance so that the attachment of ferritin antibody to cytoplasmic strands must be viewed as fortuitous

Studies with Ferritin Conjugated Antibody

Inasmuch as it proved impossible in our hands to ensure both penetration of antibody and preservation of intracellular architecture further attempts at immunological identification of cytoplasmic antigenic deposits were abandoned As a routine procedure cell suspensions were therefore merely subjected to prefixation with 3 per cent glutaraldehyde as described in Materials and Methods then incubated with ferritin tagged antibody extensively washed with PBS and prepared for sectioning in the usual manner Uninfected L cells handled in this fashion presented the appearance shown in Fig 9 The cellular membrane is well defined and ribosomes are


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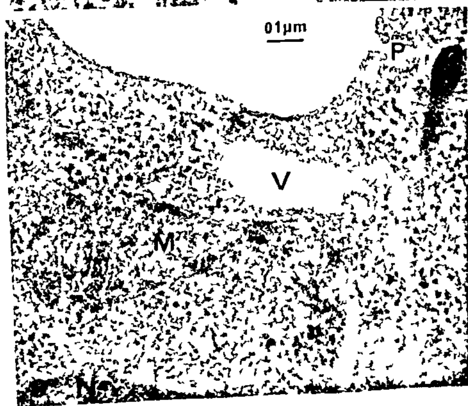

01µm
P
V
M
No

Fig 7 HeLa cell 21 hours after infection with VSV (m = 0 01) treated with ferritin tagged antibody (87 000 ×)

Fig 8 L cell 8 hours after infection with VSV (m = 10) incubated with ferritin conjugated antibody (87 000 ×)

Fig 9 Normal L cell incubated with ferritin conjugated antibody Nucleus = N mitochondrion = M vacuole = V pinocytotic vacuole = P (87 000 ×)

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diffusely distributed throughout the cytoplasm A small portion of the nucleus can be recognized in the lower left hand corner Mitochondria display clearly delimited cisternae An intracellular vacuole can be seen in the centre of the illustration and in an adjoining region another vacuole has a pinocytotic connection with the extracellular environment Ferritin labelled antibody is not in evidence

In order to expose all cells simultaneously to the inoculum a large dose of virus (n = 10) was employed in this and all subsequent experiments Groups of Blake bottles of L cells were infected as described and at 4 hour intervals up to the 20th hour duplicate bottles were harvested After appropriate handling cells were examined in the electron microscope Already 4 hours after infection some cells could be located which sprouted virus particles at the cellular surface These occurred usually in clusters and were not evenly dispersed along the circumference of the cell Fig 8 shows a portion of an L cell infected 8 hours previously with VSV An accumulation of virus particles can be seen ranged along the cellular periphery Ferritin conjugated antibody is closely associated with this area Those portions of the cell where virus is absent do not display ferritin deposits The cellular membrane is intact antibody has not penetrated into the interior of the cell which seems undisturbed By the time 16 hours had elapsed (Fig 10) discrete virus is still only seen at the cellular periphery often with ferritin granules closely outlining the contours of the particles There does not appear to be any area inside the cell which is in any specific way involved in virus replication

A portion of an uninfected HeLa cell is shown in Fig 12 which includes a small area of the nucleus Virus particles are absent and there is no non specific tagging of cellular structures with ferritin conjugated antibody As with L cell cultures infected similarly virus releasing cells could be detected already 4 hours after infection although they were located with greater ease at later intervals Fig 11 shows a cell infected 12 hours earlier with VSV Particles some of which are clearly budding from the cellular surface can be recognized Ferritin antibody is closely associated with this group of viruses but not with parts of the cell membrane where virus is not in evidence Antibody has not penetrated into the cell and the cytoplasm appears to be unaffected Infected HeLa cells did not show extensive vacuolization Yet occasionally virus containing vacuoles were observed as illustrated in Fig 13 The particles shown are partially tagged with ferritin conjugated antibody presumably because the vacuole maintained a direct contact with the exterior Ferritin granules are noticeably absent in the cytoplasm of this cell which had been infected 15 hours earlier However ferritin antibody was associated with another group of virus particles located at the cellular periphery (Fig 14) At this stage of infection of the culture conglomerates of ferritin tagged virus particles were often seen in the vicinity of cellular debris and without any close connection with other as yet intact cells in the area One may assume that these clumps of virus were derived from cells which had disintegrated either spontaneously or during handling for electron microscopy

DISCUSSION

Comparison of the multiplication patterns of VSV in L and in HeLa cells disclosed that somewhat higher yields of infective virus were elicited from the latter Moreover HeLa cultures appeared to be more rapidly destroyed


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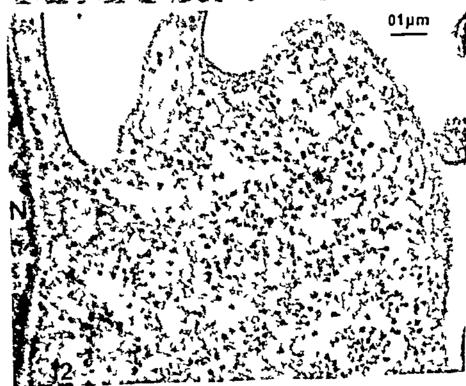
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12

Fig 10 L cell 16 hours after infection with VSV (m = 10) treated with ferritin conjugated antibody (87 000 ×)
Fig 11 HeLa cell 12 hours after infection with VSV (m = 10) treated with ferritin conjugated antibody (87 000 ×)
Fig 12 Normal HeLa cell incubated with ferritin conjugated antibody Nucleus = N (87 000 ×)

than L cells under the same conditions of infection In both systems within a few hours after initial contact with VSV fluorescent deposits of variable sizes formed in the cytoplasm Nuclei were devoid of fluorescing patches at any time during the infectious process which in L cells culminated in the formation of giant syncytia that could contain hundreds of nuclei

The scrutiny of more that 5 000 fields of view in the electron microscope uncovered no evidence that unusual cytoplasmic structures could be associated with the multiplication of VSV Within hours after infection virus identifiable by its distinctive morphology was found ranged along the cellular periphery in extracellular aggregates or in occasional intracytoplasmic vacuoles as observed by others in these and different cell cultures (4 7, 8 10 13 19 20 25) Efforts to locate with the aid of ferritin conjugated antibodies intra cellular areas which might correspond to the fluorescent antigenic patches observed in the light microscope did not meet with success Conventional techniques for introducing ferritin conjugated antibody into the cell were found to destroy cellular integrity beyond interpretation However none of the other methods used to open up cells sufficiently to permit penetration of ferritin antibody (cryostat sectioning chopping of cells digitonin dimethylsulphoxide) gave any evidence that such areas exist Non specific alterations of cellular architecture such as thickened mitochondria in L cells or circular whorl like arrangements of the endoplasmic reticulum in HeLa cells were observed on occasion dur



Figs 13 14 HeLa cell 16 hours after infection with VSV (*m* = 10) treated with ferritin conjugated antibody (87 000 ×)

ing advanced stages of infection However, such changes were noted also in uninfected cultures maintained under suboptimal conditions and they could not be associated with viral infection

Virus which was found in vacuoles generally had the appearance of fully mature forms Its presence coincided with but did not precede the appearance of viral particles at the cell boundry Occasionally hollow forms of virus were located in cytoplasmic vacuoles (Fig 4) Their relationship to the virus growth cycle was not clear since they could not be correlated with a particular stage of infection The possibility cannot be excluded that the intravacuolar VSV observed actually originated from other cells in the culture and represented a product of pinocytosis In fact, mouse peritoneal macrophages exposed to large doses of VSV for 20 to 40 minutes ingested and stored viral particles in vacuoles resembling those described (*Shechmeister* & *Birch Andersen* unpublished data)

The morphological evidence obtained favours the concept that VSV differentiates into structurally distinct particles at the periphery of L and HeLa cells in the absence of discernible intermediate developmental stages In optimal sections both outer and inner portions of viral particles were continuous with the cellular membrane and cytoplasm (Figs 7 and 11)

The extent of infection with VSV as recorded in the electron microscope falls considerably short of the dramatic involvement detected by the immunofluorescent method More specifically neither the number of the infected cells nor the progressive accumulation of viral antigen find their counterpart in corresponding electron microscopic observations The fact that one technique sums up the activities of a whole cell, and the other only that of a cellular portion does not satisfactorily resolve these discrepancies It may be suggested that preparatory handling for electron microscopy destroys those cells which are in more advanced stages of infection so that they are eliminated from the analysis On the other hand fluorescent antibody may not stain intracellular depo its of viral antigen but aggregates of virus at the cellular surface in areas contiguous to the nucleus The findings hardly permit one to chose between these or other alternatives

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EFFECTS OF THYMECTOMY UPON THE FORMATION OF FOA-KURLOFF CELLS IN THE GUINEA PIG

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The peculiar cytoplasmic inclusion body found in mononuclear cells of guinea pig blood and lymphoid tissues—the Foa Kurloff body—is believed to play an undefined role in the humoral immune mechanism of that animal On the ultrastructural level similarities between such cells and plasmocytic cells have earlier been noted In order to test an often postulated thymic origin of these cells and to evaluate the effects of an immunological ablation on their formation thymectomy was carried out on guinea pigs subsequently subjected to repeated injections of oestrogen The experiment demonstrated unequivocally that the presence in the guinea pig of thymic tissue has no influence on the rate of formation of Fóa Kurloff bodies in its tissues

The peculiar cytoplasmic inclusion body in mononuclear cells of peripheral blood and spleens of guinea pigs was originally described by *Kurloff* in 1889 and *Foa & Carbonne* in the same year Later on *Pearse* (1949) outlined its histochemical properties (a mucoprotein) and its similarity to the well known Russell body of the plasma cell The role of oestrogens in determining the number in blood and tissues of such *Foa Kurloff body* containing cells (FKB) was suggested by *Alexeieff & Joukoff* (1928) and shown by *Ledingham* (1940)

The nature of the FKB has been discussed elsewhere (*Christensen et al* 1970) As regards its origin a host of hypotheses has been proposed but still very little is known

A thymic origin has been repeatedly claimed (*Jolly & Férster* 1929 *Bimes et al* 1964 *Simmons* 1963 1965) Investigations by *Bimes et al* (1964) on the number of FKB-containing lymphocytes in the peripheral blood concluded that at least half the number of these cells were of thymic origin Recently *Simmons* (1965) produced micrographs showing FKB containing cells crowding the efferent lymphatics of the guinea pig thymus thus claiming an exclusive thymic formation of these cells

In view of the suspected participation of FKB cells in humoral immune mechanisms (*Christensen et al* 1962 1970) and the well established immunological function of the thymus we considered the relevance of investigating the effect of thymectomy on the subsequent development of FKB cells in oestrogen treated guinea pigs

MATERIALS AND METHODS

The experimental animals were 20 female guinea pigs with an average weight of 300 grammes Ten

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TABLE 1 *Results of Cellulose Acetate Electrophoresis on Sera from Thymectomized and Sham Thymectomized Oestrogen Treated Guinea Pigs*

	Thymectomized Experimental group	Sham Thymectomized Control group
Total Serum Protein (± S D)	4.44 ± 0.33 g%	4.57 ± 0.36 g%
Albumin	2.33 ± 0.26 g%	2.4[illegible] ± 0.36 g%
α 1 globulin	0.32 ± 0.07 g%	0.43 ± 0.08 g%
α 2 globulin	0.63 ± 0.07 g%	0.72 ± 0.16 g%
β 1 globulin	0.28 ± 0.05 g%	0.26 ± 0.04 g%
β 2 globulin	0.26 ± 0.07 g%	0.21 ± 0.05 g%
γ globulin	0.62 ± 0.10 g%	0.49 ± 0.13 g%

animals were thymectomized in Nembutal anaesthesia through a median incision in the neck and formed subsequently the experimental group The remaining ten animals—the controls—were likewise sham thymectomized Following this all twenty guinea pigs received a total of 6 weekly intramuscular injections of 0 25 mgs oestradiol dipropionate (Follicyclin) One week after the last injection all animals were killed by exsanguination through a heart puncture in ether anaesthesia

The following organs were removed and immediately fixed in 10 per cent neutral formalin spleen lymph nodes thymus (if any) lung liver ileum kidney and adrenals After paraffin embedding sections were cut 4 microns thick and stained with haematoxylin eosin methyl green pyronin periodic acid Schiff alkaline Congo red Alcian blue and Hale

Shortly after bleeding serum was separated and a total protein determination and electrophoresis on cellulose acetate in a veronal buffer employing standard methods was carried out

The number of FKB containing cells was estimated in all sections of tissue by counting in a number of randomly selected field of vision As a FKB was considered any sharply defined brilliantly P.A.S positive homogeneous inclusion body with an average diameter exceeding 1 micron

RESULTS

As regards organ distribution and topography within a given organ morphological appearance total number and staining properties of the numerous FKB containing cells no difference whatsoever between thymectomized and non thymectomized animals was noted In both groups an abundance of typical FKB was found throughout the tissues especially in the spleens In methyl green pyronin stained sections the pyroninophilia of the lymphoid tissues of the thymectomized guinea pigs matched that of the sham thymectomized controls perfectly None of the thymectomized animals showed any signs of lymphoid depletion Likewise the number and the distribution of plasmocytic cells were equal in both groups In both groups however the general impression of pyroninophilia at this stage was weak the picture being dominated by the heavily P A S positive FKB inclusion bodies

Similarly the electrophoretic pattern of the thymectomized group failed to show any significant difference in comparison with that of the sham thymectomized control group (*Table 1*) No reduction of the gammaglobulins in the thymectomized group appeared On the contrary a slight though not significant increase was noted

In the thymus glands of the sham thymectomized controls a moderate number of FKB containing mononuclear cells was noted in the cortical areas Only a few were recognized within thymic lymphatic vessels

DISCUSSION

The present results heavily indicate the possibility of a non thymic origin of FKB cells This assumption was anticipated in an earlier publication (*Christensen et al* 1970) in which was described ultrastructural investigations revealing the presence within the splenic reticulum of several developmental

stages of FKB-containing cells, thus pointing towards a local formation at least in that particular organ These results, however only rule out the concept of an *exclusive* thymic origin of FKB cells though substantiated by the above presented observations

The formation of FKB has been shown to be accompanied by a proliferation of RE type cells visualizing synthetizing activities somewhat along the plasmocellular line (*Christensen et al* 1970) The observation balances the findings of a simultaneous hyperimmunoglobulinaemia in FKB producing guinea pigs by *Charles & Nicol* (1961) and the electrophoretic findings in the present material is a confirmation hereof That the relative level of immunoglobulins remained unaffected in our thymectomized group was to be expected from a report of similar experiments in rats (*Andersen & Bierring* 1964) The significance of the rather slight hyperglobulinaemia accompanying the formation of FKB is unknown The two events may be totally unrelated the rise in gamma globulin being an independent response to a slight immunogenic action of the oestrogen Or they may reflect a participation of the FKB cells in an as yet unknown type of immunological performance

In the adult animal the immunological consequences of thymectomy are confined to the cellular or delayed type of immune reactivity (*Miller* 1962 *Miller et al* 1963 *Andersen & Bierring* 1964) The fact that the presence of thymic tissue in the guinea pig has no influence on the subsequently induced formation of FKB compared to the observations of morphological similarities between FKB cells and cells of the plasmocytic series may suggest a role of the former cell type in the humoral immune mechanisms of the guinea pig

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TYPES OF PNEUMOCOCCI FOUND IN BLOOD, SPINAL FLUID AND PLEURAL EXUDATE DURING A PERIOD OF 15 YEARS (1954–1969)

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The frequency of pneumococcal types in blood spinal fluid and pleural exudate during the years 1954–69 has been compared with the corre ponding numbers for 1939–47 The frequency of types within the groups is given for the years 1962–69

At present there are 82 known types of pneumococci (*Lund & Munksgaard* 1967) Practically all the pneumococci isolated from patients hitherto have been found to be sensitive to penicillin Thus typing of pneumococci would not be expected to be of any particular clinical interest Nevertheless still more laboratories perform typing

Statens Seruminstitut is the only laboratory that produces diagnostic Pneumococcus sera covering all 82 types Interest in these sera has been increasing in many countries during the last 6–7 years

The numbers of pneumococci typed at Statens Seruminstitut have already been published for the years 1940–42 and for 1939–47 (*Mørch* 1944 1949) In order to find out whether the frequency of the different types has changed corresponding figures are given here for the past 15 years (1954–69) Since the establishment of the new types Pn 48 (*Lund* 1962) and Pn 12A (*Lund & Munksgaard* 1967) Statens Seruminstitut has been able to type all capsulated pneumococci received from Denmark and other countries

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TABLE 1 *Comparison of the Most Common Pneumococcal Types in 1939–47 and in 1954–69*

Material	1939–1947	1954–1969
Blood	1 2 3-6-4 12 23	1-4 14-7 3-23-8
Spinal fluid	1 3-6-18-2-4 19	18-14 7-6-3-19- 3
Pleural exudate	1 2 6 3 19 7 8	3-1 23-14 19 9-7

Figures indicate the Pneumococcus types or groups found in patients during the years in question

Table 1 shows a comparison between the most common types of pneumococci isolated from patients in the years 1939–47 and 1954–69 In 1939–47 type 1 was the most frequent in blood spinal fluid and pleural exudate During that period type 2 and 6 were among the predominant types while in 1954–69 the same types were found less frequently

In 1954–69 (Table 2) type 1 was the type found most frequently in blood and the second most frequent in pleural exudate but it was relatively rare in spinal fluid being number 13 in frequency During the same period type 18 was the one found most often in

1944, *Lund* 1969) Thus, if it is intended to vaccinate against the most common types of pneumococci, it will be difficult to choose the types

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ENDOGENOUS INCORPORATION OF ^{3}P IN *NEISSERIA MENINGITIDIS*

1 *The Effects of CO_2 and Electron Flux*

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Meningococcal extracts mediate an incorporation of ^{3}P in the presence of Mg and ADP which is improved by the addition of EDTA A major part of this incorporation is due to a polynucleotide phosphorylase activity and is independent of electron flux But the endogenous respiration is also accompanied by a phosphorylation When the cytochrome oxidase is inhibited by KCN phosphorylation occurs concomitantly with the endogenous reduction of added mammalian ferricytochrome c This phosphorylation is nearly doubled when $KHCO_3$ is included in the system It has not been possible to demonstrate an oxidative phosphorylation on the electron oxygen transport level in meningococcal extracts with NADH NADPH or ferrocytochrome c as electron sources The phosphorylation connected with electron flux and its enhancement by $KHCO_3$ have been discussed in relation to the CO_2 requirements typical for *N meningitidis*

Several functions may be required in order to establish exogenous metabolism and growth when a starved microorganism is transferred to a new growth medium Conceivably biosynthesis of adaptive enzymes must occur before available extracellular substrates may be utilized as a source of energy and building material Energy is also required for osmotic regulation and maintenance of intracellular pH values Furthermore proper red ox potentials must be established for the oxidative reactions to proceed Endogenous metabolism may thus be considered to fulfil several vital functions These are to serve as a source of energy to provide substrates for the synthesis of adaptive enzymes and degraded cellular constituents to perform special functions such as furnishing a source of reducing power (2)

Initiation of growth in *Neisseria meningitidis* on minimal media requires fairly large inocula (4) The reasons are not at all clear but it has been postulated that a pronounced interdependence is established between individual units in a culture of meningococci and that this phenomenon may result in great demands to derepression and induction in order that growth may start from individual colony forming units (6) The requirements to endogenous metabolism may thus be very great in *N meningitidis* in order to initiate growth from small inocula

The energy metabolism in *N meningitidis* has been examined in this laboratory by measuring the oxidation of various substrates (5 7 11) and the phosphorylation of ADP

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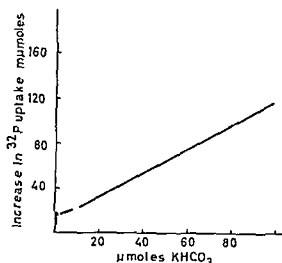


Fig 1 Effect of $KHCO_3$ on the incorporation of ^{32}P The complete system contained ADP 5 μmoles ^{32}P ortopho phate 1 6 μmoles $MgSO_4$ 10 μmoles EDTA 2 μmoles Meningococcus extract 0 5 ml $KHCO_3$ as indicated and 0 05 M Tris buffer pH 7 4 to a total volume of 2 ml The reaction was stopped after 30 min at 24 C by the addition of 0 5 ml 25 per cent TCA and analysed for ^{32}P incorporation

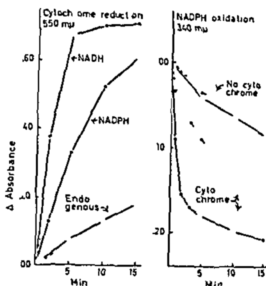


Fig 2 Reduction of ferricytochrome c and oxidation of reduced pyridine nucleotide coenzymes in the presence of meningococcal extracts Experimental conditions as in Table 3 Experiments with $KHCO_3$ present (————) and with no added $KHCO_3$ (- - - -)

TABLE 3 *Incorporation of ^{32}P in the Presence of Meningococcal Extracts and Ferricytochrome c*

Experimental conditions		^{32}P Uptake in mμmoles			
		Expt 1 (Extract no 115)		Expt 2 (Extract no 116)	
		No electron donor	NADPH	No electron donor	NADH
$KHCO_3$ absent	Extr + Cytochr	85 88	80 46	44 77	43 5?
	Extr	80 71	81 47	36 97	39 08
	Effect of Cytochr	5 17	--	7 80	4 44
$KHCO_3$ present	Extr + Cytochr	101 40	102 60	51 54	49 57
	Extr	91 96	91 83	37 93	40 46
	Effect of Cytochr	9 44	10 77	13 61	9 11

The complete system contained ADP 5 μmoles ^{32}P 3 2 μmoles $MgSO_4$ 10 μmole EDTA 2 μmoles KCN 1 μmole $KHCO_3$ 1 per cent 0 1 ml Ferricytochrome c 0 06 0 07 μmole NADH (or NADPH) 0 25 μmole Meningococcus extract dilution 0 2 ml 0 05 M Tris buffer pH 7 4 to a total volume of 2 ml Reduction of cytochrome c as well as oxidation of NADH or NADPH was followed in the spectrophotometer (Fig 2) The reactions were stopped after 15 min by the addition of 0 5 ml 25 per cent TCA and analysed for the incorporation of ^{32}P

electron transport a cytochrome effect is constantly present There is some variation between different extracts (Table 3) but the results with each extract may easily be reproduced The cytochrome effect is strongly enhanced by the addition of $KHCO_3$ In each of these experiments the reduction of cytochrome c was measured and calculated

in terms of ½ ΔFe^{III} cytochrome c mμ moles equal to mμmoles O (8 13) The cyto chrome reduction could then be related to the cytochrome effect on phosphorylation The calculations show that the P/O ratio of Experiment 1 of Table 3 changes from 1 05 to 1 85 upon the addition of $KHCO_3$ and that of Experiment 2 from 0 59 to 0 94

The more rapid reduction of cytochrome c from NADH and NADPH does not result in an increased phosphorylation On the contrary the phosphorylation is reduced sig nificantly under these conditions both in terms of total ³²P uptake and in relation to the reduction of cytochrome c The inhibit ing effect is particularly evident when no $KHCO_3$ is added

Numerous attempts have been made in this laboratory to demonstrate a true oxida tive phosphorylation on the electron oxygen transport level in *N meningitidis* These ex periments were first based on conventional manometric technique with the measurement of phosphate uptake and subsequently on the examination of ³²P incorporation during electron flow from various electron donors In agreement with the findings from the present experiments it has never been possi ble to find a phosphorylation coupled with the reduction of cytochrome c from NADH or NADPH Neither has it been possible to find phosphorylation connected with the oxidation of reduced cytochrome c (*Jyssum* & *Jyssum* unpubl results) These experi ments were based on those performed with *Alcaligenes faecalis A otobacter vinelandii Mycobacterium phlei* and *Micrococcus lyso deicticus* (3) Extracts have been prepared in several ways also in the presence of sta bilizing fluid (1) Crude extracts as well as particles separated in the ultracentrifuge (12) supplemented with various amounts of supernatant fluid have been tested

DISCUSSION

In the course of experiments concerned with phosphorylation in *N meningitidis* it was observed that the presence of carbonate in creased the endogenous phosphorylation sig nificantly There are several indications that this effect is due to a change in the endogen ous oxidative metabolism resulting in a more rapid synthesis of ATP from ADP In the presence of cyanide the effect is reduced and it is restored upon the addition of cyto chrome c as an electron acceptor

A mobilization of endogenous energy sources may evidently be obtained in *N me ningitidis* by permitting an electron flux and it is possible to make this process far more effective by supplying a sufficient concen tration of CO_2

The endogenous phosphorylation in the presence of oxidized cytochrome c and cya nide may either be considered a true elec tron transport phosphorylation or it may be the result of an increased endogenous phos phorylation on the substrate level due to the reestablishment of the electron flow The inhibition by other electron donors like NADH and NADPH of the phosphorylation under these conditions as well as the previ ous observation that the endogenous phos phorylation is not uncoupled after ageing of the extract (9) seems to indicate that a phos phorylation on the electron oxygen transport level plays no part in these experiments

In spite of numerous attempts it has not been possible to demonstrate a true oxidative phosphorylation in *N meningitidis* with elec tron donors like NADH NADPH or ferro-cytochrome c We do not know whether this implies that no such phosphorylation occurs *in vivo* or whether the results are due to some unknown technical fallacy But if it indicates that the microbe is without an energy trap on the electron-oxygen transport level it would certainly mean that meningo-cocci are handicapped with regard to the utilization of available energy sources

While the reasons for the increment in phosphorylation in the presence of $KHCO_3$ are far from clear it appears natural to dis cuss the phenomenon in relation to the ne cessity of an external supply of CO_2 (in $KHCO_3$) for the initiation of growth of this bacterium (4) Several CO dependant

fluence of various metabolic intermediates on the endogenous incorporation of ³²P in the presence of meningococcal extracts

MATERIALS AND METHODS

The methodology and experimental manipulations used in this communication were the same as those described in the preceding paper (9) The following is especially relevant to the present report

Chemical procedures Acetate ¹⁴C was counted after microdiffusion according to *Conway* (7) After diffusion at room temperature for 8½ h the content of the outer chamber of the unit was neutralized with 0 1 N Ba(OH) and samples were transferred to planchets dried and counted in a gas flow counter (4)

Acetoacetate was prepared from acetoacetic acid ethyl ester by a modification of the method of *Davies* (11 12)

The incorporation of ³²P into nucleoside phosphates was determined after isolation of the latter by chromatography and strip chart recording according to the method previously employed (8)

RESULTS

Effect of Acetate

Acetate is a metabolic end product in the oxidation of glucose by *N meningitidis* (4, 5) and is not itself significantly metabolized when added as a substrate (3) Therefore it was surprising to find that the addition of acetate to meningococcal extracts resulted in considerable phosphorylation in the presence

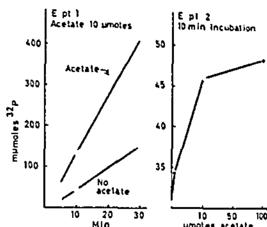


Fig 1 Effect of acetate on the incorporation of ³²P in the presence of meningococcal extracts —The complete system contained ADP 5 µmoles ³²P 1 6 µmoles MgSO₄ 10 µmoles EDTA 2 µmoles KCN 0 1 µmole Na acetate 10 µmoles meningococcal extract 0 2 ml 0 05 M Tris buffer pH 7 4 to a total volume of 2 ml The reaction was stopped with 0 5 ml 25 per cent TCA and counted wet as described under methods

TABLE 1 *Activation of the Acetate Effect by EDTA and KCN*

Expt no	Experimental system*			³²P uptake in mµmoles†			
	Acetate	EDTA	KCN	Uptake measured	Effect of acetate	Effect of EDTA	Effect of KCN
1	—		—	422 9	ND	ND	ND
	+			568 6	+ 145 7	ND	ND
	—		+	445 2	ND	ND	+ 22 3
	+	—	+	818 3	+ 373 1	ND	+ 49 7
	—	+		383 6	ND	— 39 3	ND
	+	+		817 9	+ 431 3	+ 249 3	ND
	—	+	+	422 4	ND	— 22 8	+ 38 8
	+	+	+	901 2	+ 478 8	+ 82 9	+ 83 3
2	—	+		386 1	ND	ND	ND
	+	+		951 9	+ 565 8	ND	ND
		+	+	414 9	ND	ND	+ 8 8
	+	+	+	960 3	+ 545 4	ND	+ 8 4

* + Substance present — substance absent

† ND = Not determined

The complete system contained ADP 10 µmoles ³²P 1 6 µmole MgSO₄ 10 µmol EDTA 5 µmoles KCN 0 5 µmole Na Acetate 5 µmoles Meningococcus extract 0 5 ml 0 05 M Tris buffer pH 7 4 to 1 2 ml The reaction was stopped after 30 min with 0 5 ml 25 per cent TCA Analysis and calculations as described in method

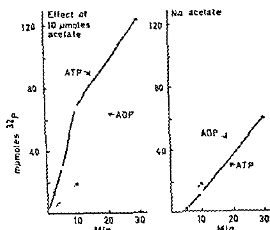


Fig 2 Appearance of labelled ATP and ADP in the presence of acetate —The experimental system was analogous to that of Fig 1 Nucleotide co factors analysed after chromatography and strip chart recording (8 9)

of ADP MgSO and ortophosphate (Fig 1) When attempts were made to reduce the transport of electrons to oxygen by cyanide no inhibition was obtained On the contrary the addition of KCN more than doubled the ³ P uptake associated with acetate (Table 1) Furthermore it was found that the addition of EDTA also enhanced this phosphory lation and that the effect of EDTA was nearly the same as that of cyanide under otherwise identical conditions It was also observed that the addition of these chemicals resulted in less variation between parallels and accordingly in less experimental error

No significant effect of acetate was ob served without the addition of ADP Fig 2 shows that ³ P is incorporated into ATP and ADP It would seem that in the presence of acetate radioactive ATP is first synthesized and that radioactivity is subsequently appear ing in ADP This is in contrast to the phos phorylation without added acetate Here ADP and ATP are approximately equally labelled from the earliest times in the way it has previously been observed during ex periments with polynucleotide phosphorylase activities in meningococcal extracts (8) These observations seem to indicate that ATP is indeed synthesized from ADP in the presence of acetate and that radioactive ADP originates as the result of an adenylate kinase activity AMP + ATP ⇄ 2 ADP (8)

The fate of ¹ C labelled acetate in the ex perimental system was examined In these experiments the quantitative composition of the system was changed in order to obtain adequate recovery of acetate (Table 2) After incubation up to 30 min and irrespective of a pronounced effect on the incorporation of ³ P no significant change in the amount of acetate was observed This seems strongly to indicate that acetate does not function as a substrate for the reaction

If acetate acts by activating an endogen ous transfer of energy from energy rich com pounds some idea as to the actual transfer system may presumably be obtained by an analysis of inhibitor effects as well as of co factor requirements The phosphorylation in the presence of acetate is strongly inhibited by NaF while the inhibition of the phos phorylation without added acetate is moder ately inhibited With increasing concentra

TABLE 2 *Eff ct of Acetate on the P Inco po ation and the Fate of the A tate*

Expt no	³ P uptake in mµmoles		¹⁴C activity in acetate in CPM	
	No acetate	Acetate	Zero time	30 min
1	461 5	1096 0	10?13	10334
2	414 9	960 3	101?8	10101

The experimental system was analogous to the complete system of Table 1 In experiments with ¹ C acetate the experiments were stopped with 0 I ml I per cent H SO_4 and analysed by microdiffusion For the determination of P incorporation the experiments were stopped with 0 5 ml 25 per cent TCA and counted wet as in the previous experiments

of no importance for this incorporation of phosphate either

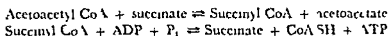
DISCUSSION

The previous communication showed that an improvement of the conditions for oxidative metabolism in extracts from *N meningitidis* results in increased endogenous phosphorylation and that this phosphorylation probably is on the substrate level (9) The experiments presented above show that the addition of certain metabolic intermediates, and even an important end product in the metabolism of this microbe such as acetate also enhances the incorporation of ^{32}P

The phosphorylation associated with acetate, succinate, propionate and acetoacetate does not seem to require an active electron transport to oxygen The ^{32}P incorporation is increased by the addition of cyanide although cyanide drastically interferes with the respiration in *N meningitidis*, primarily by inhibiting the cytochrome oxidase (6) and inhibits the endogenous phosphorylation associated with the electron transport (9) The activating effect of cyanide may be due to the formation of a chelate with a metal since the same effect seems to be obtained with EDTA The lack of inhibition by cyanide does not necessarily mean that reactions involving oxidations and reductions play no part in the phosphorylation observed since there may be a balance between the two sets of reactions and an oxidation of NADH as well as of NADPH takes place even in the presence of cyanide (9) But it certainly indicates that the ^{32}P incorporation associated with the compounds mentioned is of another nature than that connected with the endogenous electron flux to ferricytochrome c (9)

The experiments reveal two interesting properties of the uptake system For the first substances like acetate and succinate do not furnish energy for the phosphorylation of ADP to ATP because they exert their effect without being significantly broken down For the second the inhibitor effects as well as the cofactor requirements focus attention on the thiol ester linkage Therefore it appears feasable that reactions which mediate transfer of energy between CoA compounds and ATP play an important role and that the substances added activate this transfer for instance by supplying the necessary substrates for the enzyme reactions involved

This type of mechanism may be visualized in connection with the effect of succinate and acetoacetate since a coupling of succinyl CoA transferase with the succinic thiokinase could well mediate such transfer


Acetoacetyl CoA + succinate ⇌ Succinyl CoA + acetoacetate
Succinyl CoA + ADP + P_i ⇌ Succinate + CoASH + ATP

There are in the principle two ways in which such a mechanism may result in labelling of ATP The first is that the incorporation is due to an exchange of ^{32}P with the phosphate groups of the nucleotide The second is that the incorporation represents a real transfer of energy from other energy rich (storage) compounds to ATP The fact that storage of the extracts reduce the ^{32}P incorporation may be an indication that endogenous energy sources are of importance

The endogenous sources of energy in *N meningitidis* are not at all known but the present experiments certainly bring in mind the importance of acetyl CoA propionyl CoA succinyl CoA and acetoacetyl CoA in the catabolism of fats (10) The comparatively low endogenous RQ may also indicate that the substrates for the endogenous respiration are lipids (9)

One very important aspect of the present observations is the imitation of a true oxidative phosphorylation This is particularly evident in the experiments with succinate Obviously this type of ^{32}P incorporation must be taken into consideration before a phosphorylation occurring concomitantly with the oxidation of a substrate is accepted as a true oxidative phosphorylation

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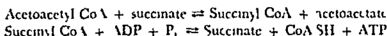
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$$\text{Acetoacetyl CoA} + \text{succinate} \rightleftharpoons \text{Succinyl CoA} + \text{acetoacetate}$$
$$\text{Succinyl CoA} + \text{ADP} + P_i \rightleftharpoons \text{Succinate} + \text{CoA SH} + \text{ATP}$$

There are in the principle two ways in which such a mechanism may result in labelling of ATP The first is that the incorporation is due to an exchange of ^{32}P with the phosphate groups of the nucleotide The second is that the incorporation represents a real transfer of energy from other energy rich (storage) compounds to ATP The fact that storage of the extracts reduce the ^{32}P incorporation may be an indication that endogenous energy sources are of importance

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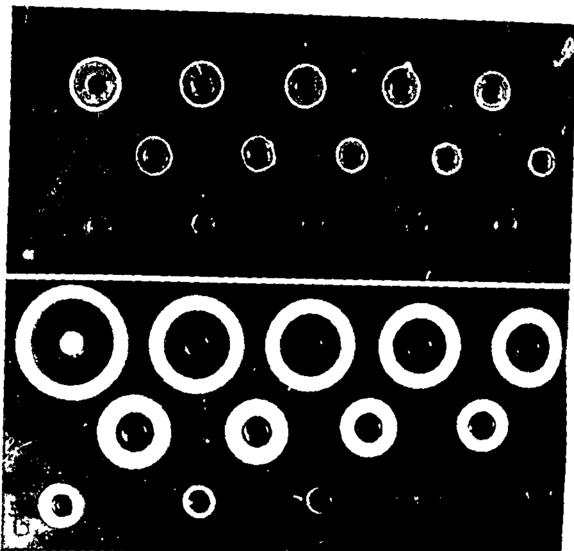


Fig 1 Precipitation zones in casein agar corresponding to different dilutions of bovine trypsin (a) and *Aspergillus oryzae* proteinase (b) a The first zone (upper left) corresponds to 1 mg trypsin per ml the next to 0 1 mg per ml and then 2 fold serial dilutions of the 0 1 mg solution b The first zone (upper left) corresponds to 30 mg *Aspergillus oryzae* proteinase per ml solution the next to 3 mg per ml and then 2 fold serial dilutions of the 3 mg solution The plates were incubated at 37 C for 18 hours

meters of the zones were measured three times and the mean value was plotted as a function of the degree of dilution (Fig 2) The resulting curve was extrapolated to the abscissa (point A in Fig 2) to find the casein precipitation titre CI titre) The amount of enzyme in the dilution corresponding to the point of intersection between the standard curve and the abscissa is defined as one *diffusion unit* A standard curve for each enzyme was drawn as the best straight line by plotting the zone diameters against the logarithms of the number of diffusion units present in 0 025 l of the dilution (Figs 3 and 4)

Serial dilutions of an enzyme were also placed in corresponding wells without and with addition of inhibitors the zone diameters were measured and the titre determined in the absence and presence of inhibitors

For qualitative detection of inhibitory activity on casein agar plates (*CI test*) *the method generally used* was to place 3 mm wide filter paper strips (Schleicher & Schull No 2043 bmgl) moistened with a solution containing the inhibitor on the agar surface and to leave the strips there for about 3 hours at 37 C. *After removal of these* strips similar strips moistened with proteolytic en

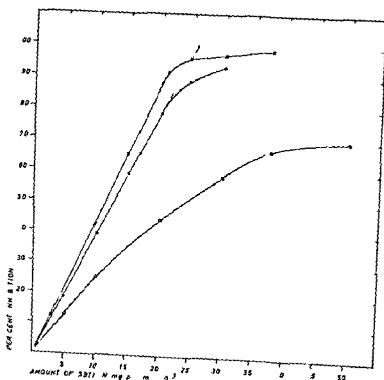


Fig 6 Per cent inhibition of bovine tryp in (x——x) swine trypsin (●——●) and α-chymotrypsin (O——O) by different amounts of SBTI as determined by the Kunitz method The amounts of en zymes used were tho e giving an increase in OD value of about 0 600 in the absence of inhibitor *i e* 0 025 mg of bovine and swine trypsins and 0 0175 mg of α chymotrypsin

diameters versus a 2 fold serial dilution of trypsin to which was added equal amounts of two solutions of OMTI before applying the mixtures into wells is presented in Fig 10 Although OMTI in concentrations of 0 1 and 1 0 mg per ml caused a decrease in the CP titre from approximately 700 to 52 and 22 CP units respectively the zone diameter for the lowest dilution of enzyme increased from 13 mm in the absence of to 17 mm in the presence of the inhibitor However by putting OMTI into one well on a casein agar plate and enzymes in adjacent wells (Fig 11 a) a flattening of the zones of bovine tryp sin was seen similar to that which occurred for swine trypsin and *Bacillus subtilis* protein ase while the zone caused by α chymotrypsin was not affected

When the inhibitory effect of SBTI upon α chymotrypsin was tested by the CPI test relatively high concentrations ... mg per ml and low concentrations of the enzyme had to be used in order to detect inhibition (Fig 5) Inhibitor concentrations lower than 1 mg per ml and enzyme concentrations higher than 500 CP units per 0 025 ml failed to produce inhibition zones When the enzyme and in hibitor were placed in adjacent wells a flat tening of the precipitation zone also occurred in this system (Fig 11 b)

DISCUSSION

The comparison between the sensitivity of the CP test and the Kunitz method for de tection of proteolytic enzyme activity shows that the first method is much more sensitive than the latter If an increase in the OD val ue of 0 05 or more is considered necessary for obtaining significant results by the Kunitz method ... Limabean trypsin ... about

TABLE 2 *Effect of the Same Inhibitors towards the Same Proteolytic Enzymes as Shown in Table 1 and Fig 5 Tested by the Kunitz Method Amount of Enzymes Used is that Giving an OD Value at 280 mμ of about 0.600 under the Assay Conditions Used in the Absence of Inhibitor*

Inhibitors	Trypsin (bovine) M W 24000	Trypsin (swine) M W 24000	α-chymotrypsin M W 24000	*Bacillus subtilis* proteinase	Ficin	*Aspergillus oryzae* proteinase
OMTI§ M W 24000–28800	Linear inhibition† Wi/We* = 1.2 Mol i/Mol e‖ = 1	Linear inhibition Wi/We = 1.3 Mol i/Mol e = 1	—	2 per cent inhibition at Wi/We = 1	–	
SBTI M W 20000–24000	Linear inhibition Wi/We = 1 Mol i/Mol e = 1	Linear inhibition Wi/We = 1 Mol i/Mol e = 1	Non linear inhibition Wi/We = 4 at 50 per cent inhibition Mol i/Mol e = 4 at 50 per cent inhibition	–	–	
LBTI M W 8290–9890	Linear inhibition Wi/We = 0.4 Mol i/Mol e = 1	Linear inhibition Wi/We = 0.5 Mol i/Mol e = 1	Non linear inhibition Wi/We = 8 at 50 per cent inhibition	–	–	
PTI M W 6500	Linear inhibition Wi/We = 0.26 Mol i/Mol e = 1	Linear inhibition Wi/We = 0.32 Mol i/Mol e = 1	Non linear inhibition Wi/We = 0.5 at 50 per cent inhibition Mol i/Mol e = 4 at 50 per cent inhibition	–	–	
Trasylol	Linear inhibition	Linear inhibition	Non linear inhibition	Slight inhibition using 0.050 ml	–	4 per cent inhibition by 0.05 ml

§ The same abbreviations as in Table 1

† Linear inhibition Linearly related to the enzyme concentration up to 50 per cent inhibition or more

* Wi/We Weight of inhibitor to weight of enzyme

‖ Mol i/Mol e Molecules of inhibitor to molecules of enzyme

– No inhibition observed

Fig 9 shows the corresponding effect of different concentrations of OMTI upon different concentrations of the same enzymes as in Fig 8 In this case an increase in opacity and even a widening of the zone of precipitation occurred when relatively high concentrations of bovine trypsin were used although this was not the case for swine [illegible] and more pronounced precipitation occurred where application of the inhibitor and enzyme overlapped was seen with both of the two different preparations of bovine trypsin used Wider zones were also obtained in the presence rather than in the absence of the inhibitor when mixtures of certain concentrations of bovine trypsin and OMTI were [illegible] in wells in casein agar plates. Zone

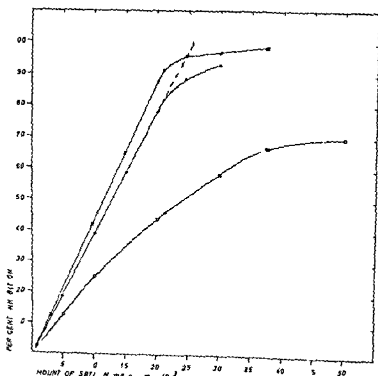


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GROWTH RATES AND VIRULENCE OF *E COLI* IN EXUDATES PRODUCED DURING INTESTINAL STRANGULATION OBSTRUCTION IN RATS

TORE MIDTVEDT and ARNE TRIPPESTAD

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When 0 5 ml of a suspension of *E coli* (5×10^7 per ml) in the sterile filtrate of late strangulation fluid was injected intraperitoneally on mice all the mice died within 24 hours None of the mice injected with the same numbers of microbes suspended in "early strangulation fluid filtrate or in a casein medium died during this period *E coli* cultured in media containing early or late strangulation fluid or the casein medium did not vary significantly in rates of growth or virulence A direct effect on the bacteria by substances in the late strangulation fluid filtrate did not appear to represent a factor of importance for the virulence enhancing property of the late filtrate

During intestinal strangulation obstruction an exudate accumulates in the peritoneal cavity This exudate is called strangulation fluid Amundsen & Midtvedt (1) collected strangulation fluid at intervals from rats subjected to strangulation obstruction of a segment of the lower ileum They found that fluid collected later than 24 hours after operation was lethal when injected into the peritoneal cavity of normal mice in amounts of 0 5 ml per 25 g All the lethal fluid portions were heavily contaminated with bacteria When lethal strangulation fluid was sterilized by filtration the lethal effect was abolished The lethal property was regained when viable *E coli* were suspended into the filtrate The number of *E coli* required to kill the assay mice was however smaller when the bacteria were suspended in such filtrate than when other suspending media were used such as normal saline rat plasma or filtered fluid produced during the first 24 hours of the ailment

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The virulence enhancing effect of the strangulation fluid filtrate might be due to stimulation of bacterial multiplication, alteration of the pathogenicity of the bacteria or influence on host defence factors The present investigation was carried out in attempt to correlate the virulence enhancing and growth promoting activities of such filtrates on *E coli*

MATERIALS AND METHODS

Bacteria

The strain of *E coli* was the one used in experiments presented in another report in which the culture technique was also described (9) The bacteria were grown in a medium prepared according to *Benacerraf et al* (2) without the addition of lactated phosphate This medium is called casein medium in the following

Strangulation Fluid

was collected from 10 male and 10 female of a local strain The rats weighed between 2?0 and 450 g The technique used was described by *Amundsen & Midtvedt* (1) The strangulation fluid was pooled, filter sterilized and stored at

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T-MYCOPLASMAS IN THE GENITO-URINARY TRACT OF THE FEMALE

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Cervical and urethral specimens from 247 females were cultured for T mycoplasmas The occurrence of T mycoplasmas in clean voided mid stream and catheterized urine specimens was also investigated There was no significant difference in the recovery rate of T mycoplasmas in the genito urinary tract between healthy non pregnant women and women with signs of genital infections (45 8 and 55 4 per cent respectively) In healthy pregnant females the incidence of T mycoplasmas was significantly higher or 68 4 per cent In women taking oral contraceptives T mycoplasmas were found more frequently than in women of comparable ages and groups not taking oral contraceptives Prepubertal girls and postmenopausal women had a low incidence of T mycoplasmas (8 0 and 4 3 per cent respectively) T mycoplasmas were cultured from 15 4 per cent of catheterized urine specimens collected from women harbouring these organisms in the urethra while the corresponding figure in clean voided mid stream urine specimens was 84 9 per cent This study indicates that T mycoplasmas occur as commensals in the female genital tract The hormonal status of the individual seems to be an important factor for their occurrence T mycoplasmas often occur in urine specimens as contaminants from the urethra or the vulva Sampling from the urethra or the cervix is more reliable than collection of urine for the demonstration of T mycoplasmas in the female genito urinary tract

Since Shepard s first report of T strain *Mycoplasma* in 1954 an aetiological relationship of T mycoplasmas* to non gonococcal urethritis in the male has been discussed by several workers However the role of T mycoplasmas in this condition has remained dubious (*Black & Rasmussen* 1968)

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* To conform with recent recommendations (*Taylor Robinson et al* 1968) the T strain *Mycoplasma* will be called T mycoplasmas in this paper

In contrast to the interest taken in T mycoplasmas in the male few report have treated their occurrence in the female genito urinary tract *Shepard et al* (1964) cultured T mycoplasmas from 61 per cent of female consorts to men with non gonococcal urethritis *Csonka et al* (1966) and *Archer* (1968) frequently isolated these organisms from the urine of females in the childbearing age It has been suggested that T mycoplasmas may be involved in reproductive failure (*Kundin et al* 1967) and premature birth (*Klein et al* 1969)

The present study was undertaken to in

the group of non pregnant women with clinical signs of infection (Group III), T mycoplasmas were demonstrated in 55 4 per cent

There was no significant difference in the occurrence of T mycoplasmas between the non pregnant women in the childbearing age with and without signs of genital infection ($X = 0.94$ $p > 0.3$) On the other hand, there was a highly significant difference in the isolation rate of T mycoplasmas between the menstruating women on the one hand, and the children and the postmenopausal women, on the other hand ($X = 10.60$ $p < 0.01$ $X^2 = 12.38$ $p < 0.001$) The difference in the incidence of T mycoplasmas between pregnant and non pregnant females in comparable age groups was less significant ($X^2 = 5.55$ $0.02 > p > 0.01$)

In addition to the females presented in Table 1, 24 women taking oral contraceptives were investigated T mycoplasmas were recovered from 14 women or in 58 3 per cent

Except for the 49 pregnant women in whom the clinical examination revealed normal conditions, another 14 pregnant women with signs of infection in the lower genital tract were investigated Cultures yielded growth of T mycoplasmas in 12 of these women A more abundant growth was generally obtained from these infected pregnant women than from any other of the examined groups

In 90 per cent of the investigated cases in which T mycoplasmas were recovered from the urethra, they were also cultured from the cervix. In this respect no difference was observed between the groups.

Isolation of T mycoplasmas from Urine Specimens

The results of cultures from clean voided mid stream urine specimens or catheterized urine and urethral samples in the same individuals are shown in Table 2 Clean voided, mid stream specimens were obtained from 117 women in the childbearing age T mycoplasmas were cultured from 40 of the specimens, or in 34 2 per cent The corresponding figure for cultures from the urethra in these patients was 59 or in 50 4 per cent

From the healthy non pregnant women in the childbearing age from whom T mycoplasmas were isolated from the urethra clean voided urine specimens yielded growth of T mycoplasmas in 60 per cent The corresponding figure for the pregnant women was 90 per cent From the urine of 21 postmenopausal women and 16 children T mycoplasmas were isolated from one sample in each group

From 30 patients catheterized urine specimens were collected From these specimens T mycoplasmas were recovered in two instances From the urethra of these patients T mycoplasmas were isolated in 16 of the cases

TABLE 2 *Isolation Rate of T Mycoplasmas in the Urethra and*

	Healthy females (Group I)†				
		T mycoplasmas isolated			
		Urine		Urethra	
	Number N	n	n per cent of N	n	n per cent of N
Voided urine specimens	31	9	29 0	15	48 4
Catheterized urine specimens	8	1	12 5		50

† Children and postmenopausal women are not included

Quantitative cultures for T mycoplasmas were made from 152 urine specimens Thirty five of the urine specimens contained $< 10^3$ CFU per ml 12 of the specimens 10^3 to 10^5 CFU per ml and eight of the specimens $> 10^5$ CFU per ml From the remaining samples no growth was obtained T mycoplasmas were demonstrated in three patients with $\geq 10^5$ CFU per ml urine in two or more samples collected consecutively From 61 clean voided mid stream urine specimens, cultures were made from uncentrifuged urine as well as from the supernatants and the deposits after centrifugation The uncentrifuged specimens yielded growth in 13 instances the deposits in 12 instances and the supernatants in 11 instances The number of CFU obtained on cultures from the uncentrifuged samples and from the deposits was similar

T Mycoplasmas in Relation to Doderlein s Bacillus and Candida albicans

In women from whom cultures had been made for T mycoplasmas vaginal smears were collected for staining procedures T mycoplasmas were recovered from 48 per cent of the women where the vaginal flora morphologically was predominated by Doderlein s bacillus From women where the vaginal smears showed a mixed bacterial flora T mycoplasmas were cultured in 61 per cent

The medium used for isolation of T mycoplasmas was also suitable for cultivation of *Candida Candida albicans* was isolated from 41 of the women in the present study No difference was demonstrated in the isolation rates of T mycoplasmas between these women and women in comparable groups and ages from whom *Candida albicans* was not cultured

Recovery of T Mycoplasmas after Antibiotic Treatment

Seven patients with genital infections harbouring T mycoplasmas were treated with 1000 mg of chloramphenicol and 2 500 000 i u of penicillin administered orally daily for two weeks Control cultures after treatment yielded growth of T mycoplasmas in five of the patients All of them still presented clinical signs of infection Fifteen patients with genital infections from whom T mycoplasmas were cultured were treated with 600 mg of methacycline per day for eight days The organisms were isolated from 11 of these patients after treatment In this group no clinical signs of infection were found after treatment Another two patients were treated with 1000 mg of erythromycin orally daily for eight days These two patients harboured T mycoplasmas before but not after the treatment

*[illegible] d d or Catheteri ed Urine Specimens in the Same Individuals**

Pregnant women (Group II)					Women with genital infections (Group III)				
Number	T mycoplasmas isolated				Number	T mycoplasmas isolated			
	Urine		Urethra			Urine		Urethra	
N	n	n per cent of N	n	n per cent of N	N	n	n per cent of N	n	n per cent of N
55	25	45 5	28	50 9	31	6	19 4	16	51 6
					17	1	5 9	11	64 7

* Urine specimens were not obtained from 9 women in G oup I 2 women in Group II and 7 women in Group III

4

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FINE STRUCTURE AND TAXONOMIC POSITION OF *NEISSERIA HAEMOLYSANS* (THJØTTA & BØE 1938) OR *GEMELLA HAEMOLYSANS* (BERGER 1960)

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Five bacterial strains labelled either *Neisseria* or *Gemella haemolysans* were studied with the electron microscope They were found to be mutually alike and to possess a fine structure like that generally described for Gram positive microorganisms The mean moles per cent guanine + cytosine of the strains was 33 5 which is significantly different from the values hitherto obtained in the Gram negative *Neisseria*

Electron microscopy of a Gram variable bacterial culture labelled *Neisseria haemolysans* (ATCC 10379) showed a Gram positive type of cell wall and an inner structure and mode of division resembling those of typical Gram positive organisms (*Reyn et al* 1966) Previously *Berger* (3 4) had found that *N haemolysans* deviated to such an extent from *Neisseria* as defined by *Breed Murray* & *Smith* (7) that it could not be retained in this genus A new genus was proposed to which the generic name *Gemella* was given *G haemolysans* was described as a Gram negative aerobic oxidase and catalase negative genus within the *Neisseriaceae* By gas chromatography *Yamakawa* & *Ueta* (43) found that the contents of fatty acids and sugars of *N haemolysans* (ATCC 10379) differed from that of other *Neisseria* studied (*N meningitidis N gonorrhoeae N catarrhalis N flavescens N perflava N subflava*) These *Neisseria* contained large amounts of even numbered fatty acids as are generally found in other Gram negative bacteria

The present investigation was performed in order to collect further data on the fine structure and other characteristics of *N* or *G haemolysans* which differ from those of the true *Neisseria* In addition the moles per cent guanine + cytosine (GC) of the strains were determined and compared with those of the true *Neisseria*

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MATERIAL AND METHODS

Strains

1 a *N haemolysans* (ATCC 10379) Obtained directly from the American Type Culture Collection

1 b *N haemolysans* (ATCC 10379) Obtained from B W Catlin Milwaukee Wisconsin U.S.A.

1 c *G haemolysans* (NH Berger) Obtained from U Berger identical with NCTC 5414 the authentic strain and with ATCC 10379

2 *G haemolysans* (NCTC 10243)

11 *Kundsin R B Driscoll S G & Ming P M L* Strain of mycoplasma associated with human reproductive failure Science 157 1573–1574 1967

12 *Mårdh P A & Westrom L* Tubal and cervical cultures in acute salpingitis with special reference to Mycoplasma hominis and T strain Mycoplasma Brit J vener Dis 1970

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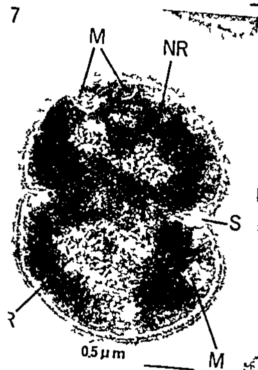
20 *Taylor Robinson D Williams M H & Haig D A* The isolation and comparative biological and physical characteristics of T mycoplasmas of cattle J gen Microbiol 54 33 46 1968

6

NR

NR

0.50µm



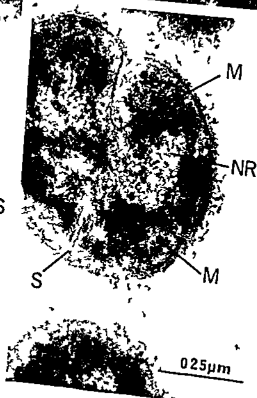


TABLE 1 *Determination of Moles Per Cent GC by the Method of DNA Buoyant Density (Mandel 1966) Calculated Using the Schildkraut et al Equation 1962*

Organism	Buoyant density	Mean per cent GC	
1 a	1 692 1 694	33 7	
1 c	1 693 1 695	34 7	
2	1 693 1 692	33 2	Grand Mean
3	1 692 1 693	33 2	± σ
4	1 693 1 690	32 1	33 5 ± 1 6
5	1 695 1 692	34 2	

37° C better on agar enriched with ascitic fluid than on plain broth agar, no growth at 10° C or 45° C Beta haemolysis on rabbit and horse blood agar after about 3 days (except no 5) Growth on modified HYL medium resulted in greening or yellowing of this medium (except no 5) Oxidase and catalase negative Nitrites not produced from nitrates, nitrites reduced by three strains (1 b 2, 3) Acid produced from glucose fructose maltose sucrose glycogen and starch No acid from lactose galactose arabinose mannitol sorbitol inulin, xylose raffinose No polysaccharide production from sucrose Very weak growth in Hugh & Leifson s medium acid not produced from glucose in this medium under anaerobic conditions Methyl red and Voges Proskauer tests positive Aesculin not broken down No hydrolysis of arginine No growth on medium with tellurite Indole not produced No formation of H S gelatinase or urease Resistant to optochin Sensitive to sodium penicillin G, streptomycin tetracycline sulphathiazole (except no 5), chloramphenicol erythromycin spiramycin and ristocetin Rods were not observed after provocation with penicillin Analysis of lactic acid production from glucose was not attempted

Moles Per Cent GC

The results are presented in Table 1

Electron Microscopy

The fine structure as revealed by electron microscopy showed that cell wall cytoplasmic membrane *mesosomes* cytoplasm ith ribosomes and nuclear material of the v rious cultures were very much alike as w also the pattern of division However ce *size and* shape varied a little from one culture to another, *this applied also to the amount of ex*tracellular slimy material Structural details of each of the five *Gemella* strains are presented in Figs 1–13 and 15 of which a description is given below Figs 14 and 16 how the fine structure of a typical Gram positive coccus and a typical Gram negative coccus, respectively (*S faecium* and *N gonorrhoeae*)

Figs 1 3 6 9 and 10 demonstrate in a moderate magnification (43000 ×) the overall features of strains nos 1 a 1 c 2 3 and 4 Two of the strains (Figs. 1 and 6) were grown in BBA and harvested in the logarithmic phase of growth, the 3 other cultures were harvested from solid media after 18–20 hours of growth The cells grown in fluid media were better preserved than those grown *on solid medium General appearance cocci* approximately 0 5 by 0 5–0 6 μ Most cells are surrounded by a corona of flocular material suggestive of a slimy layer or microcapsule The outline of *the cell wall* is even three zones are just distinguishable in the wall which varies somewhat in thickness and density a narrow dense inner zone a somewhat wider less dense intermediate zone and a rather narrow but more dense outer zone to which the flocular material adheres The *plasma membrane* appears at this low magnification as a narrow

Fig 6 Strain 3 Note especially nuclear stru ture and sparse amount of flocculer material. M gnification 43000 ×

Figs 7 and 8 Strain 2 Note numer us wl rled m sosomes and septum formation Magnification 90000

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DISCUSSION

The lacking evidence of C type particles (1 2 5) in malignant cells from these plasma cell neoplasms is surprising since such RNA virus (22) is responsible for many induced lymphatic (15 21) and myeloid (13) leukaemias in mice and negatively stained preparations of serum from some mice used for preparation of the subcellular extracts (10) showed virus like particles (25)

A possible explanation is a very low virus concentration in the neoplastic tissue an assumption which is consistent with our findings of a very long latent period when transferring the present neoplasms with subcellular material (9)

Until now intracisternal A type particles are the only virus-like particles observed in our plasma cell neoplasms. The size and substructure of the particles are very similar—though not identical—to that displayed by A type particles in other plasma cell neoplasias (4 18 24)

The encountered morphological differences between A type particles from different sources are such as a varying amount of electron dense material on the particle surface or absence/presence of electron dense granules inside the shells It is not possible to decide on the significance of these morphological variations because they may be secondary to the varying fixation and embedding procedures employed by the different investigators

It is of interest that our demonstration of electron dense granules in the particle core may lend some support to the biochemical demonstration of RNA intrinsic to the shells of A type particles (19)

A type particles have previously been described in murine plasma cell neoplasias induced in BALB/c mice by mineral oil (4 18 24) As transfer of such neoplasias with subcellular material was unsuccessful the A type particles were considered not to be oncogenic viruses (4 20 24)

A type particles have furthermore been found in methylcholantrene induced sarcomas (14) in Ehrlich s ascites tumour cells (12) and together with C type particles in various leukaemia neoplasms (3 6 7 17) several of which are known to be induced by C type particles (3 6 17 23)

Despite the fact that A type particles thus are commonly observed in various neoplasms their possible biological activities are still unknown and it has never been proved that they—e g as precursors of C type particles—in any way are related to the cellular production of leukaemia virus (16 19) This implies the conclusion that the present electron microscopic investigation gives no evidence as to which agent possibly might be responsible for the subcellular transfer of our plasma cell neoplasias

Another statement to be made is that A type particles observed in our plasma cell neoplasias obviously have no connection with the paraprotein production of these tumours since particles occur with the same frequency in neoplasms with and without paraprotein production

This investigation was supported in part by grants from *Anders Hasselbalch Fond* til Leukæmiens Bekæmpelse *Daell Fonden Rask Orsted Fonden* and *Allan Wibergs Legat* til Stotte for Leukæmi Forskning The Institute of Tumor Virus Research is supported by grants from the *Einar Willumsen Memorial Fund* and the *Danish Cancer Society*

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To 50 ml of the base was added 50 ml of a 4 per cent glucose solution and 76 ml distilled water and 1 M KH_2PO_4 and/or 1 M K_2HPO_4 according to the following schedule

	KH_2PO_4	K_2HPO_4
pH 5	24	–
pH 6	21	3
pH 7	9 5	14 5
pH 8	–	24

Each test was made *in duplo* After 12–15 days growth the pH of the medium was 5 9–6 5 *in* cultures started at pH 5 (4 experiments) *in cultures* started at pH 6 around 6 5 *in cultures* started at pH 7, around 7 2 *in cultures started* at pH 8 around 7 8 – The weight of the mycelium was estimated after the mycelium had been dried for 24 h at + 60 C

Deep fermentation The fungus was cultivated in a 10 litres laboratory fermentor (Biotec Type FL 110) at a stirrer speed of 325 r p m Extra aeration (3–8 1/min) was provided by means of a gas sparger with capillary inlet Midland Silicones Antifoam F G in a concentration of 0 p p m was used in some experiments (in culture under standard conditions the antifoam substance did not interfere with the accumulation of EPF in the culture medium) The standard casein hydrolysate medium (*Wallerstrom* 1969a) was used it was sterilized by autoclaving *in situ* in the fermentor except for the glucose component which was sterilized by Seitz filtering and added afterwards The original pH of the medium was 5 Mycelia from two 250 ml volume shake cultures grown for 6–13 days served as inoculum The mycelia were washed twice in physiological saline in order to prevent transfer of preformed EPF with the inoculum Samples of culture fluid were taken from the fermentor twice a day and studied for their content of EPF and cultured on blood agar plates to exclude infection with bacteria or moulds To check the quality of the inoculum control 250 ml-cultures were started simultaneously from the same inoculum with standard technique

Counter current separation Extracts of EPF were treated in a Craig apparatus (E-C Apparatus C Swarthmore PA) containing 50 tubes A mixture of cyclohexane and benzene (75 25) equilibrated with water was used as the organic phase The process was run for 5 6 h after which the organic phase of the tubes was evaporated and the water phase extracted with 5 ml ethyl acetate Paper discs 12 mm in diameter were imbibed with the solvent and dried and afterwards tested for antibiotic activity in the agar diffusion test against the standard test strain of *Staph aure* The contents of tubes with antibacterial activity were pooled pH of the fluid was adjusted to 5 and it was extracted 3 times with ethyl acetate and finally evaporated to dryness at about 35 C in vacuum.

Thin layer chromatography (ascending) was undertaken to purify EPF and to compare it with fucidin and 3 keto-fusidic acid It was performed on dichlorofluorescein impregnated silica gel ((Antec Trageplatten SL 254 200 × 200 mm Antec AG Birsfelden) with diethyl ether – glacial acetic acid (100 1) as the solvent system Counter-current fractionated EPF and the antibiotics to be compared with it were applied as diethyl ether solutions and the chromatograms were run for about 45 minutes *i e* until the front had nearly reached the top of the plate The antibiotics appeared as dark spots in UV light their antibiotic activity was checked against the staphylococcal strain Material collected from the spot was again subjected to thin layer chromatography with the same system

Paper chromatography (ascending) for classifying EPF *and comparing* it with fucidin was *carried out with the* system and methods described by *Betina (1964)* Strips of Whatman No 1 paper *1 × 35 cm were used* and the spots of antibiotic *were applied 3* cm from the lower end of the strip The antibiotics were applied as acetone solutions *the amount of fucidin* on each strip was 0 25 mcg *and the amount of EPF* 2 5 units (for definition of unit see *Wallerstrom* 1969a) For the devel*opment of the* chromatograms 500 ml cylindric *glass jars* were used The strips were immersed in *the solvent to* a depth of 1 cm The development was *stopped when* the solvent front had migrated 15 *cm from the origin* The following solvent systems were used

1 Distilled water
2 n Butanol saturated with water
3 Ethyl acetate saturated with water
4 Bensene saturated with water
5 3 *per cent* NH_4Cl *in water*
6 Isoamyl acetate—*methanol—99 per cent* formic acid – water (65 20 5 10)
7 *n* Butyl *acetate—methyl ethyl ketone*—0 15 M *phosphate buffer pH 7 4* (60 25 5)
8 Ethyl acetate n hexane – 0 15 M phosphate buffer pH 6 0 (65 15 20)

After the chromatogram had been run the strips were tested by the bio-autographic method *i e* placed on the surface of agar plates inoculated with the test strain of *Staph aureus* After incubation overnight at 37 C the position of the antibiotic spots could be determined from the zones of inhibition in the bacterial growth.

pH-chromatography ad modum *Betina* (1965) was performed with Whatman No 1 paper strips that were saturated with buffer solutions at pH levels from 2 to 1? and dried. The buffer systems used were for pH 2 3 and 4 sodium ci-

trate – HCl for pH5 and 6 sodium citrate – NaOH for pH 7 and 8 KH_2PO_4 – K_2HPO_4 for pH 9 10 11 and 12 glycocoll – NaOH Ethyl acetate was used as eluent The size of the strips the amount of antibiotics placed on them and the chromatographic development were as described above

Chemical colour reactions Samples of EPF purified by countercurrent separation followed by a double run on thin layer chromatography were examined for their colour reaction with a solution of $SbCl_3$ in chloroform (the system was slowly heated to about 100 C and kept at that temperature for 30 min) and were subjected to the Salkowski reaction (sulphuric acid was made to form a layer under a solution of the substance in chloroform) and Liebermann Burchard s reaction (addition of a chloroform solution of acetic anhydride to a solution of the substance in concentrated sulphuric acid the reaction was made at refrigerator temperature) For comparison samples of fucidin were examined at the same time

The susceptibility of EPF to alkalinization was compared with that of fucidin and cephalosporin P_1 Acetone solutions of the three antibiotics were evaporated cooled to +4 C and afterwards treated with 2 N NaOH After 30 minutes the solutions were re acidified (pH 5) by addition of 1 N HCl The activity of the solution against *Staph aureus* was assessed before and after the treatment

RESULTS

1 *Culture in Buffered Medium*

The investigation showed that when the culture media was kept below neutral pH a relatively dense growth of mycelium was obtained while the concentration of EPF in the medium was much lower than when the reaction was maintained at alkaline pH The results of a typical experiment are given in Fig 1

In this experiment the final dry weight of the mycelium per 100 ml of culture medium was 974 mg for cultures started at pH 5 for those started at pH 6 908 mg at pH 7 765 mg at pH 8 697 mg

The weight of the mycelium recovered in medium with an addition of buffer was invariably less than when the fungus was cultivated in standard medium (*Wallerstrom* 1969 a) where it was on the average 1050 mg per 100 ml culture The tendency to a

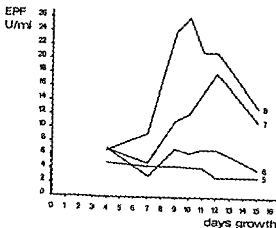


Fig 1 Concentration of EPF in cultures with buffer solution added to the medium (Figures to the right indicate pH of the buffer solution added)

better formation of antibiotic at higher pH despite lower weight of mycelium was noted in all the experiments (altogether five)

2 *Cultivation in Laboratory Fermentor*

Cultivation of *E floccosum* in a laboratory fermentor in volumes of 7 to 10 litres gave abundant growth of mycelium and as a rule it was necessary to discontinue the experiment after 20–54 hours growth because the density of the mycelium made further culture difficult The amount of EPF in the culture medium was small and never exceeded 10 U/ml The controls (250 ml cultures grown on a rotary shaker or with a magnetic stirrer) gave much better results, and the final concentration of EPF after 5–6 days growth ranged between 25 and 100 U/ml A number of experiments with other types of fermentors gave equally poor yields even when cultivation was extended to 10 to 12 days

3 *Chemical Colour Reactions*

In order to find out whether EPF was a steroid Salkowski s reaction was perform[illegible] and a so called reverse Salkowski rea[illegible] was obtained the sulphuric acid which [illegible] med a layer under the chloroform solu[illegible] EPF took on a dark blood red [illegible] same result was obtained with

To 50 ml of the base was added 50 ml of a 4 per cent glucose solution and 76 ml distilled water, and 1 M KH_2PO_4 and/or 1 M K_2HPO_4 according to the following schedule

	KH_2PO_4	K_2HPO_4
pH 5	24	–
pH 6	21	3
pH 7	9 5	14 5
pH 8	–	24

Each test was made *in duplo*. After 12–15 days growth the pH of the medium was 5 9–6 5 *in* cultures started at pH 5 (4 experiments), *in cul*tures started at pH 6 around 6 5, *in cultures* started at pH 7 around 7 2, *in cultures started* at pH 8 around 7 8. – The *weight of the mycelium* was estimated after the mycelium had been dried for 24 h at + 60 C

Deep fermentation The fungus was cultivated in a 10 litres laboratory fermentor (Biotec Type FL 110) at a stirrer speed of 325 r p m Extra aeration (3–8 l/min) was provided by means of a gas sparger with capillary inlet Midland Silicones Antifoam F G in a concentration of 20 p p m was used in some experiments (in culture under standard conditions the antifoam substance did not interfere with the accumulation of EPF in the culture medium) The standard casein hydrolysate medium (*Wallerstrom* 1969a) was used it was sterilized by autoclaving *in situ* in the fermentor except for the glucose component which was sterilized by Seitz filtering and added afterwards The original pH of the medium was 5 Mycelia from two 250 ml volume shake cultures grown for 6–13 days served as inoculum The mycelia were washed twice in physiological saline in order to prevent transfer of preformed EPF with the inoculum Samples of culture fluid were taken from the fermentor twice a day and studied for their content of EPF and cultured on blood agar plates to exclude infection with bacteria or moulds *To check the quality of the inoculum control 250 ml-cultures* were started simultaneously from the same inoculum with standard technique

Counter current separation Extracts of EPF were treated in a Craig apparatus (E-C Apparatus C Swarthmore PA) containing 50 tubes A mixture of cyclohexane and benzene (75 25) equilibrated with water was used as the organic phase The process was run for 5 6 h after which the organic phase of the tubes was evaporated and the water phase extracted with 5 ml ethyl acetate Paper discs 12 mm in diameter were imbibed with the solvent and dried and afterwards tested for antibiotic activity in the agar diffusion test against the standard test strain of *Staph aure* The contents of tubes with antibacterial activity were pooled pH of the fluid was adjusted to 5 and it was extracted 3 times with ethyl acetate and finally evaporated to dryness at about 35 C in vacuum.

Thin layer chromatography (ascending) was undertaken to purify EPF and to compare it with fucidin and 3 keto-fusidic acid It was performed on dichlorofluorescein impregnated silica gel C (Antec Trageplatten SL 254 200 × 200 mm Antec AG Birsfelden) with diethyl ether – glacial acetic acid (100 1) as the solvent system. Counter-current fractionated EPF and the antibiotics to be compared with it were applied as diethyl ether solutions and the chromatograms were run for about 45 minutes *i e* until the front had nearly reached the top of the plate The antibiotics appeared as dark spots in UV light their antibiotic activity was checked against the staphylococcal strain Material collected from the spot was again subjected to thin layer chromatography with the same system

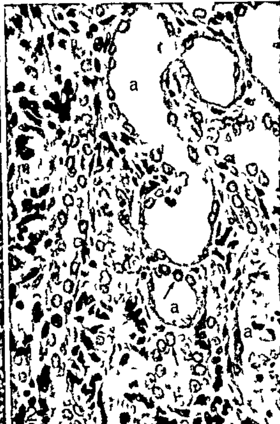
Paper chromatography (ascending) for classifying EPF and comparing it with fucidin was *carried out with the system* and methods described by *Betina (1964)* *Strips* of Whatman No 1 paper 1 × 35 *cm* were used and the spots of antibiotic *were applied 3 cm from the lower end of the strip* *The antibiotics* were applied as acetone solutions *the amount of fucidin* on each strip was 0 25 mcg *and the amount of EPF 2 5* units (for definition of unit see *Wallerstrom* 1969a) For the devel*opment of the chromatograms* 500 ml cylindric *glass jars were used The strips* were immersed in *the solvent to a depth of 1 cm* The development was *stopped* when the solvent front had migrated 15 cm from the origin The following solvent systems were used

1 Distilled water
2 n Butanol saturated with water
3 Ethyl acetate saturated with water
4 Bensene saturated with water
5 3 per cent NH_4Cl in water
6 Isoamyl acetate—methanol—99 per cent formic acid – water *(65 20 5 10)*
7 n Butyl acetate—methyl ethyl ketone—0 15 M phosphate buffer pH 7 4 (50 25 5)
8 Ethyl acetate n hexane – 0 15 M phosphate buffer pH 6 0 (65 15 20)

After the chromatogram had been run, the strips were tested by the bio-autographic method *i e* placed on the surface of agar plates inoculated with the test strain of *Staph aureus* After incubation overnight at 37 C the position of the antibiotic spots could be determined from their zones of inhibition in the bacterial growth.

pH-chromatography ad modum *Betina* (1958) was performed with Whatman No. 1 paper strips that were saturated with buffer solutions at pH levels from 2 to 12 and dried The buffer systems used were for pH 2 3 and 4 sod in cr




a
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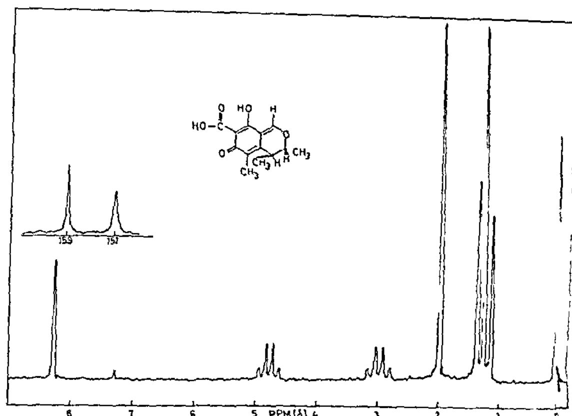


Fig 6 NMR spectrum of (–)citrinin determined on a JEOL C-60HL instrument Solvent $CDCl_3$ internal standard $(CH_3)_4$ Si The signals at δ 15 1 and δ 15 9 representing the hydroxyl group proton and the carboxyl group proton respectively disappear on deuteration

or no epithelium other tubules are collapsed with clusters of cells surrounded by thickened wavy basement membranes (Figs 7 8 9)

Further it was planned to administer 33 mg of citrinin/100 g rat during an experimental period of 14 days but all the rats died on the third day after administration 9 5 mg/100 g rat (Table 2) In the stomach of one rat haemorrhages were found in the fundus Histologically acute tubular alterations in the form of pyknotic nuclei formation of hyaline droplets and hyaline cylinders were observed

B II *Administration of Sodium Oxalate*

Administration of fraction no 2 consisting of 32 mg of oxalic acid/100 g rat did not cause any kidney damage (Table –) However as oxalic acid and oxalates are known to have nephrotoxic properties, an experiment was carried out in which large amounts of sodium oxalate were administered (see Table 3)

At necropsy the rats *a* and *b* were emaciated the kidneys were enlarged with an uneven grey brown surface On the cut surface a radial striation was noted Focal haemorrhages were observed in the fundus ventriculi from rat *a*

It was intended to administer 450 mg of sodium oxalate to rat *c* during the experimental period but the rat died after one day having received 123 mg/100 g rat The stomach was dilated and filled with a thin red brown liquid Further submucous and subserous haemorrhages in the stomach were observed By histological examination of the kidneys from rat *a* and *b* alterations in the form of dilation of the tubules, formation of

TABLE 3 *Administration of Oxalates*

Experiment	Animal	Period of time days	Type of oxalate	Amount of oxalate mg/100 g animal		Kidney damage
				single dose	total	
A I	3 rats	14	unknown type in biomass	15*	105	chronic degeneration nuclear alterations
A II	3 rats	1	unknown type in biomass	69 0*	69 0*	degeneration Ca-oxalate crystals in cortex
B I	3 rats	18	acid	5	3?	no alteration
B II	1 rat(a)	11	Na-oxalate	54	163	chronic degeneration Ca-oxalate crystals no nuclear alterations
B II	1 rat(b)	11	Na-oxalate	88	263	
B II	1 rat(c)	1	Na-oxalate	123	123	acute nephrosis Ca-oxalate crystals
+	1 pig	42	Na oxalate	100	4200	slight interstitial fibrosis

\+ According to *Hasselager* (1970)
* Determined as oxalic acid

cysts and proliferation of connective tissue especially along the medullary rays were observed A few calcium oxalate like crystals were scattered in the cortex

The kidneys from rat *c* were sites of an acute tubular nephrosis especially in the proximal tubules in the form of hydropic degeneration focal loss of brush border and desquamation of cells

Numerous needle like double refractive crystals of calcium oxalate forming rosette clusters were observed in the proximal tubule causing compression and destruction of the epithelium (Fig 10) Nuclear alterations were not observed in any of the kidneys from rat *a* *b* and *c* as found after administration of nephrotoxic barley biomass and citrinin

DISCUSSION

The nephrotoxic properties of oxalic acid and oxalates are wellknown Table 3 summarizes experiments of oxalate administration The oxalate induced kidney damage does n[illegible] clude tubular nuclear alterations. Th[illegible] rats oxalates are not able to reproduce [illegible] typical kidney degeneration which is [illegible] served after administration of neph[illegible] barley barley inoculated with *P* [illegible] or citrinin In pigs big amounts of ox[illegible] produced only a slight interstitial fibr[illegible] the kidneys (*Hasselager* 1970)

Our results obtained by administ[illegible] citrinin to animals are summarized in T[illegible] together with previously published inv[illegible] tions by other authors

Citrinin was discovered in 1931 by He[illegible] ington & *Raistrick* who isolated th[illegible] pound from several strains of *P* [illegible] Thom That the species P[illegible] includes citrinin producing members [illegible] confirmed by *Verona* & *Gambogi* [illegible] *Sakai* (1955) *Udagawa et al* (1955 (1957) and *Mirchink et al* (1966

Also other species of *Penicillium* citrinin producing members Thus [illegible]

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COMPARISON OF ANTIGENIC STRUCTURE AND PHAGE PATTERN WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM DOGS AND PIGEONS

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Seventy five strains of coagulase-positive staphylococci isolated from the nostrils of healthy dogs and 67 strains isolated from the nostrils of pigeons have been typed both serologically and with the standard set of human phages as well as with selected bovine phages Biochemically 10 of the dog strains were of the human biotype All of these were typable *serologically and* 8 were sensitive to the phages used. Sixty three dog strains and all the pigeon strains showed to a great extent biochemical properties corresponding to the canine biotype Of the whole group of these white-pigmented isolates only one strain of pigeon origin was typable by phage The dog strains and the pigeon strains differed serologically Eighty five per cent of the true canine strains isolated from dogs were easily typable The dominant antigen was a_2 and there were only few antigenic patterns. Of the pigeon strains only 24 per cent were typable In agreement with other investigations the present investiga*tion shows that* canine strains can quite successfully be typed serologically whereas the human and bovine phage sets are of little use This reflects a fundamental difference between the two typing systems

The carrier rate of *Staphylococcus aureus* in the nostrils of healthy dogs and the incidence of staphylococcal infections in dogs have been studied by many authors (22 15, 20, 12 19 21) The canine biotype of *Staph aureus* has certain biochemical characteristics which permit a distinction from the human and bovine biotypes (15, 16 17, 20, 21, 9)

Phage typing of canine strains has not been successful Only a few per cent of the strains have been found typable using the international phage typing set active against human strains and then mostly in concentrated phages (4 2 13 6 5, 20 23) The susceptibility to phages active against bovine staphylococci was even lower (4 6 5) On the other hand phages isolated from lysogenic *Staph aureus* strains of canine origin have proved to be more active (4 6 5 3 11)

In the few investigations carried out on serological typing of canine staphylococci the results have been more promising *Live & Nichols* (1965) were able to type serologically 81 per cent of 100 strains of phage un-

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typable staphylococci isolated from the nasal passages of dogs *Hahn* & *Blobel* (1968) found 67 per cent of 293 strains from infections in dogs typable *Pillet et al* (1968) could only type 23 out of 51 canine strains from cutaneous lesions using their method for serological typing In an absorbed serum produced against a canine stain (61218) 40 of the strains were agglutinated *Shimizu* (1968) was not able to agglutinate any out of 145 canine strains in 7 factor sera whereas 97 per cent of the strains agglutinated in a polyvalent serum produced against a canine strain

To our knowledge no material of any size of *Staph aureus* strains isolated from pigeons has been examined serologically or by phage typing

MATERIAL AND METHODS

By means of swabs 75 strains of staphylococci were isolated from the nares of healthy dogs from different areas of Czechoslovakia Sixty seven strains were isolated from pigeons (*Columba livia forma domestica*) which lived free in the town of Olomouc before capture When the birds had been killed samples were taken from the nasal cavities after the skin had been disinfected and the infraorbital sinuses incised

Biochemical examinations were carried out to demonstrate the formation of coagulase the production of acid from mannitol and glucose under anaerobic conditions the production of alpha beta and delta haemolysins the production of pigment egg yolk factor phosphatase protease gelatinase and urease the presence of clumping factor the type of growth on agar with crystal violet and the sensitivity to antibiotics (penicillin streptomycin chloramphenicol tetracycline erythromycin kanamycin bacitracin vancomycin and lincomycin) as well as to sublimate (9)

The strains were typed serologically using the method of O d ng (1957) For technical details see also *Haukenes* (1967) The strains were agglutinated on slides using live bacteria and 14 absorbed rabbit immune sera a_1, a b_1 c_1 e h_1 h_2 $h_1 h_2$ k k_2 k_1 m n *263 1* *263 2* The set of standard strains of *Staph aureus* used for the production of immune sera had been isolated from human sources

The absorbed serum 61218 (Lot 69 A) was obtained through the courtesy of Dr *J Pillet* Institut Pasteur Annexe de Garches The *Staph aureus* strain used for immunization was isolated from a dog (19)

Twenty four phages obtained from the Staphylococcal Reference Laboratory Colindale and the Swedish phage KS6 were used The phage typing technique of *Blair* & *Williams* (1961) was employed The strains resistant to lysis by phages at RTD were then re typed with 1 000 × RTD The following phages active against bovine staphylococci were obtained from Dr *Davidson* Agriculture Central Veterinary Laboratory Weybridge England AC_1 102 107 1563/14 S_1 and S_8 A phage isolated at the Gade Institute Bergen from a lysogenic strain of *Staph aureus* of ovine origin (208) was used in addition

RESULTS

Of the 75 isolates from dogs 63 strains had the biochemical characteristics of *Staph aureus* of canine origin (Table 1) They grew in white colonies on agar with full fat milk as well as on agar with crystal violet they produced only beta and delta haemolysin They coagulated bovine plasma but only irregularly human plasma They fermented mannitol slowly showed a weak production of the egg yolk and the clumping factor and were sensitive to all antibiotics tested and to sublimate Two strains designated as intermediate showed considerably lower biochemical activity and were closely related to the species *Staph epidermidis* The remaining 10 isolates corresponded to the biotype of human origin They produced orange pigment grew on crystal violet agar in either orange or violet colonies coagulated human but not bovine plasma fermented carbohydrates quickly, produced especially alpha and delta haemolysin and usually fibrinolysin produced the egg yolk factor intensively and were resistant to penicillin some of them even to sublimate

The 67 strains of *Staph aureus* isolated from pigeons were biochemically a quite homogeneous group very closely related to the strains that occur in dogs The strains obtained from pigeons differed from those from dogs only by a complete absence of the clumping factor a somewhat quicker fermentation of carbohydrates a more intensive coagulase reaction in human plasma, a more frequent production of alpha haemolysin and a lower protease activity (9)

TABLE 1 *Biochemical Characteristics of 142* Staph aureus *Strains of Dog and Pigeon Origin*

Biochemical properties		Origin and number of strains — Dogs 75 — Biotype and number of strains: Canine 63	Intermediate 2: Str P3	Intermediate 2: Str P33	Human 10	Pigeons 6 — Canine 6
Mannitol fermentation		slow	slow	—	fast	slow
Coagulation of plasma	human	var	—	—	+	var
	bovine	+	—	weak	—	+
	porcine	+	—	weak	+	+
	avian	+	+	—	+	+
Clumping factor		var	—	—	+	—
Haemolysin	alpha	—	—	—	+	var
	beta	+	—	—	var	+
	delta	+	—	+	+	+
Fibrinolysin		—	—	—	+	—
Pigment	orange	—	—	—	+	—
	white	+	+	+	—	+
Egg yolk factor		weak	—	weak	+	weak
Crystal violet agar test	orange	—	—	—	var	—
	violet	—	+	—	var	—
	blue white	—	—	—	—	
	white	+	—	+	—	—
Antibiotic resistance		—	T	—	T	—
Sublimate resistance		—	—	—	var	—

T = tetracycline P = penicillin

Sixty five (87 per cent) of the 75 strains of staphylococci isolated from the nares of healthy dogs could easily be typed serologically (Table 2) Of the 63 strains of canine biotype 53 were typable 10 untypable The two intermediate strains and all the 10 strains of human biotype were typable serologically Forty nine strains had a strong a_5 antigen and in addition a few other moderate or weak minor antigens Of these strains 47 belonged to the canine biotype and two were intermediate strains (P3 a_5/h P33 a_5/k_1) Six strains belonging to the canine biotype lacked the a_5 antigen

TABLE 2 *Serological Typing of 75 Dog Strains*

	Antigenic pattern	No. of strains
63 strains of canine biotype and 2 intermediate strains	$a_5/c_2/k_1$	19
	a_5/k_1	13
	a_5	9
	a_5/other antigens	8
	k_1	4
	$h_1/m/$'63 *I*	1
	c_1/k_1	1
	NT	10
10 strains of human biotype	a_5/other antigens	
	Different patterns without a_5 antigen	8
Total		5

Of the 10 dog strains which according to their biochemical characteristics corresponded to human staphylococci only 2 strains had

TABLE 3 *Serological Typing of 67 Pigeon Strains*

Antigenic pattern	No of strains
a_5	4
a_5/k_1	1
$a_5/c_1/h$	1
a_4 alone or combined with other antigens	5
Other patterns	5
NT	51
Total	67

the a_5 antigen In other respects however they differed serologically from the main group of canine strains Eight strains had quite other antigenic patterns and were all different serologically

In contrast to the dog strains only 24 per cent of the 67 strains of *Staph aureus* isolated from the nostrils of pigeons were typable serologically (Table 3) Of the 16 typable strains only 5 agglutinated strongly whereas the agglutinations of 11 strains were weak One strain (a_5 k_1) was similar to the type found in the dog material Five more strains had the a_5 antigen but here it was weak and these strains were not identical serologically with the dog strains

According to serological typing the dog strains and the pigeon strains are thus entirely different

All the strains were then agglutinated in *Pillet s* absorbed serum against the dog strain 61218 Forty eight of the 65 dog strains of canine biotype and the two intermediate strains agglutinated in this serum compared to 49 strains in the a_5 serum (Table 4) Agglutination in these sera was not due to the same antibody as a number of the strains agglutinated in the absorbed 61218 serum but not in the a_5 serum, and vice versa Also the absorbed 61218 serum shows that the 10 strains of the human type are different serologically from the true canine strains isolated from the dogs

None of the 67 pigeon strains and none of 15 *Staph aureus* human standard strains or 21 strains of bovine origin agglutinated in absorbed serum 61218

Absorbed serum 61218 thus also clearly separates the dog strains of the canine biotype from the dog strains of the human biotype and the strains of pigeon origin

Of the 75 dog strains only 8 strains were typable by phage (Table 5) One strain was typable in RTD 7 strains in 1 000 × RTD These strains were lysed by phages from group I and/or group III and 4 of them also by bovine phages The 8 typable strains all be

TABLE 4 *Agglutination of Dog Strains in Absorbed 61218 Serum and in a_5 Serum*

	Serum	No of positive reactions
63 strains of canine biotype and 2 intermediate strains	61218	50
	a_5	49
	61218 and a_5	36
	61218 (not in a_5)	14
	a_5 (not in 61218)	13
10 strains of human biotype	61218	1
	a_5	2

TABLE 5 *Phage Typing of 75 Dog Strains and 67 Pigeon Strains*

Origin	Biotype	No	Results of phage typing
Dogs	Canine	63	No strain typable
	Intermediate	2	
	Human	10	8 strains typable, 1 in RTD 7 in 1 000 × RTD
Pigeons	Canine	67	1 strain typable in 1 000 × RTD

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OCCURRENCE OF CANDIDA IN ORAL LEUKOPLAKIAS

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Biopsies from 235 patients with oral leukoplakias were investigated (83 speckled leukoplakias and 152 homogeneous leukoplakias) A candidal invation was found in 23 per cent of the leukoplakias (in 61 per cent of the speckled leukoplakias and in 3 per cent of the homogeneous leukoplakias) Epithelial atypia was associated with concurrent Candida in 61 per cent of the speckled leukoplakias *while epithelial atypia occurred in 88 per cent of the* speckled leukoplakias which were not affected by Candida

Recent years have brought several reports of an associated *Candida albicans* infection in patients with oral leukoplakia and it has been suggested that some clinical forms of leukoplakia are the result of invasion by hyphae of *Candida albicans* (1 2 3, 4)

The purpose of the present retrospective investigation has been 1) to estimate the frequency of candidal hyphae within the epithelium of speckled leukoplakias and of homogeneous leukoplakias and 2) to determine how often the presence of hyphae in a leukoplakic lesion is associated with epithelial atypia

MATERIAL AND METHOD

The source of material for the present study was *patients with oral leukoplakia seen in the Dental* Department University Hospital and the Department of Oral Surgery Royal Dental College Copenhagen during the years 1956 to 1966 The material consisted of biopsies taken with a knife in local analgesia from 235 patients 154 males and 81 females Their ages ranged from 25 to 90 years *the average being 56 years One biopsy from each* patient was taken before any treatment was performed Periodic acid Schiff stained sections with and without prior digestion with diastase and haematoxylin and eosin stained sections were examined

83 patients (53 males and 30 females with the average age of 55 years) had speckled leukoplakia while 152 patients (101 males and 51 females with the average age of 57 years) had homogeneous leukoplakia

DEFINITIONS

Leukoplakia In the present study oral leukoplakia was defined as a white patch or plaque not less than 5 mm in diameter which could not be removed by rubbing and which could not be classified as any other diagnosable disease Thus the use of the term leukoplakia did not carry any histological connotation

Speckled leukoplakia This type of leukoplakia was described by *Pindborg et al* (5) in 1963 It is a lesion with the characteristics of white patches or sometimes nodules on an erythematous background This simultaneous occurrence of white and *red areas endowes the affected* mucosa with a speckled appearance

Homogeneous leukoplakia This term was used for all other clinical types of leukoplakia

Candidal invasion Was considered when the

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Vorkommen koagulasepositiver Staphylokokken im Tierreich II Differenzierung der koagulasepositiven Staphylokokkenstamme aus dem Nasenrachenraum gesunder Tiere Zbl Bakt. I Abt Orig *202* 351–363 1967
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PHAGE TYPING OF *STAPHYLOCOCCUS AUREUS* FROM BOVINE MASTITIS

A Comparison of Phages According to Davidson to the Conventional Phage Set

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A provisional phage set suggested by Davidson for typing of "bovine" Staphylococcus aureus was compared to the international phage set in the typing of 225 strains isolated from bovine mastitis in 61 herds in central Sweden At RTD 44 9 per cent of the strains were typable with the provisional phage set and 30 7 per cent with the international set Corresponding figures at 1000 × RTD were 74 7 and 69 8 per cent With both sets a total typability of 79 1 per cent was achieved The following phage set proved most useful 29 52 A 84 3 A 6 53 75 77 42 D 107 1363/14 78 S 1 S 6 883 54 71 81

Phage typing has considerably increased our ability to define a strain of *Staphylococcus aureus* The technique was first systematized by *Wilson et al* (14) and further developed by *Williams et al* (13) The phage set was at the beginning not completely standardized but subsequently an international phage set for the differentiation of staphylococci of human origin has been agreed upon (10)

Staphylococci isolated from cattle show different biological properties (11) Some of them have the same characteristics as staphylococci of human origin but a great many of them differ from these amongst other things by their production of β toxin lack of staphylokinase their dissimilar antigenic structure and sensitivity to bacteriophages (7 9 11 12) The international phage set is not very well selected for these strains Attempts have therefore been made to modify it in order to make it more suitable for the typing of staphylococci isolated from cattle (3 4 5 6) *Davidson* (4) has constructed a set of phages by selecting 9 phages from the international phage set and adding to them 8 isolated from bovine strains of staphylococci For the time being it is called the provisional phage set and has been tested to a certain extent (2 6 9)

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The purpose of the present investigation was on the one hand to test the provisional phage set on staphylococci isolated from bovine mastitis in Sweden and on the other to try to find out whether this phage set offered any advantages over the international phage set in the differentiation of these staphylococci

MATERIAL AND METHODS

Strains The staphylococci consisted of 225 coagulase positive strains isolated from 225 cases of bovine mastitis in 61 herds in central Sweden in 1967

Phage typing The phages used were 1) the 17 phages of the provisional phage set according to *Davidson* 29 52 A AC 1 84 3 A 6 53 75 77

visional phage set might prove necessary following results of tests in various countries a considerable advance seems to have been made towards the achievement of an international phage set for staphylococci isolated from cattle

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MORAXELLA BOVIS ISOLATED FROM CATTLE WITH INFECTIOUS KERATOCONJUNCTIVITIS

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Ten strains of *Moraxella bovis* were isolated from cases of infectious bovine keratoconjunctivitis On primary isolation on blood agar they formed flat rough colonies In subculture rapid dissociation to smaller smooth convex colonies occurred This is not in agreement with the colony morphology described in the literature according to which smooth colonies appear to be predominant in primary isolation One of the strains examined was non haemolytic Examined strains did not grow under anaerobic conditions and showed dependence on high oxygen pressure Optimum growth was shown at an oxygen pressure slightly below that of atmospheric air There was no growth at 15 C and 40 C All strains were catalase negative and oxidase positive Carbohydrates were not attacked None of the strains examined decomposed urea reduced nitrate or formed hydrogen sulphide Coagulated serum and gelatin are liquefied In litmus milk alkaline peptonization took place characteristically progressing by the formation of three zones There was no growth on MacCorkey agar or on potato infusion agar The results of the antibiotic sensitivity tests are shown in Table 1 The sensitivity of the bacterium to penicillin is particularly emphasized

The clinical condition known as infectious bovine keratoconjunctivitis is probably due to various aetiological factors The organism most frequently isolated from cases of this disease is *Moraxella bovis*

In 1915 *Mitter* isolated *Micrococcus lanceolatus* and a diplobacillus which was identified as the *Morax Axenfeld* bacillus from an outbreak of contagious ophtalmitis in cattle From cases of infectious keratitis *Allen* (1919) isolated a bacterium which had important characteristics in common with the organism isolated independently by *Morax* (1896) and *Axenfeld* (1897) from cases of infectious human conjunctivitis *Jones & Little* (1923) described 24 cases of acute infectious ophthalmitis in cattle and in all cases a characteristic diplobacillus was demonstrated In 1937 *Hauduroy et al* classified the organism isolated by Jones & Little as *Haemophilus bovis* Later *Lwoff* (1939) included it in the genus *Moraxella* The bacterium does not resemble *Haemophilus* morphologically neither is it dependent on haematin (X factor) or diphosphopyridine nucleotide (V factor)

In Denmark the first clinical description of the disease was given by *Morkeberg* (1920) In the autumn of that year there had been

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TABLE 1 *Antibiotic Sensitivity of Strains of* Moraxella bovis

Strain	Sulf mm mcg/ml	Pen mm I U./ml	Strep mm mcg/ml	Chlor mm mcg/ml	Tetra mm mcg/ml	Neo mm mcg/ml	Poly mm
1	38 (0 55)	39 (0 01)	29 (0 07)	37 (0 11)	29 (0 05)	31 (0 0012)	4
2	41 (0 30)	39 (0 01)	30 (0 05)	39 (0 07)	29 (0 05)	31 (0 0012)	24
3	40 (0 38)	37 (0 02)	30 (0 05)	38 (0 08)	25 (0 22)	29 (0 0021)	25
4	45 (0 15)	39 (0 01)	30 (0 05)	38 (0 08)	27 (0 11)	31 (0 0012)	25
5	41 (0 30)	38 (0 02)	30 (0 05)	40 (0 05)	27 (0 11)	31 (0 0012)	26
6	41 (0 30)	39 (0 01)	29 (0 07)	38 (0 08)	30 (0 04)	31 (0 0012)	25
7	41 (0 30)	38 (0 02)	30 (0 05)	38 (0 08)	28 (0 08)	32 (0 0006)	23
8	42 (0 25)	41 (<0 01)	32 (0 02)	33 (0 30)	26 (0 16)	32 (0 0006)	[illegible]
9	40 (0 38)	38 (0 02)	35 (0 01)	39 (0 07)	31 (0 03)	36 (<0 0005)	25
10	36 (0 80)	40 (0 01)	31 (0 03)	37 (0 11)	29 (0 05)	31 (0 0012)	24

* The regression between diameter of inhibition zone and minimum inhibitory concentration is [illegible] calculated According to zone diameter the strains are grouped as sensitive

Results are given as diameter of inhibition zone in mm and minimum inhibition concentration (IC_{50}) in brackets

perature for growth of *M bovis* is 36° C Among the strains examined, 7 tolerated 50° C for 30 min No organism survived 50° C for 40 min or 55° C for 5 min

Biochemical Reactions

All the strains examined were catalase negative according to the methods used and in this respect there was no difference between smooth and rough phases of any strain A positive oxidase test was a constant characteristic None of the strains fermented the following monosaccharides glucose arabinose *fructose galactose, mannose rhamnose* and xylose The bacterium grew well in Hugh & Leifson s medium but there was no oxidative or fermentative decomposition of glucose The reaction in litmus milk was alkaline peptonization, beginning at the top of the medium Three zones developed the bottom zone had the normal colour of litmus milk the medium zone which was rather narrow was slightly darker the upper zone was transparent and reddish violet in colour The peptonization progressed without prior visible coagulation The rate of peptonization was dependent on the size of the inoculum In case of copious inoculation complete peptonization may take place in the course of 24–48 hours whereas in case of smaller inoculum the process may take 3–8 days or longer The characteristic 3 zones reaction in litmus milk occurred only on inoculation with an appropriate amount of material and the tube must not be shaken during incubation.

None of the strains examined decomposed urea reduced nitrate or produced hydrogen sulphide There was no growth on Simmons citrate MacConkey agar or potato infusion agar All the strains liquefied coagulated serum Gelatin was liquefied slowly at 22 C. The reaction on gelatin agar was positive after 24 hours at 37° C

Antibiotic Sensitivity

The results of the antibiotic sensitivity tests are shown in Table 1 Generally the examined strains of *M bovis* were very sensitive to the antibiotics used for the tests On estimation of their clinical effect they all were referred to group I (sensitive) i e the least inhibitory concentration is approximately ¼ of the concentration of the antibiotic which may be expected to be present in serum of patients who have been given ordinary doses as universal treatment The great sensitivity to penicillin should be particularly emphasized as this antibiotic primarily inhibits Gram positive bacteria The sensitivity to penicillin is in accordance with *Henriksen*

(1952) description of the genus *Moraxella* according to which he states that most strains are highly to moderately sensitive to penicillin

DISCUSSION

The present communication is the first description of *M bovis* isolated from cattle in Denmark

Bergey's Manual (1957) places *M bovis* together with *M lacunata* and *M liquefaciens* in the genus *Moraxella* which belongs to the family *Brucellaceae* In the course of the years a number of new species have been included in the genus Serological tests have shown pronounced cross reaction in agglutination and immunodiffusion between the species *M lacunata M bovis* and *M nonliquefaciens* although there are signs of a certain heterogeneity *Holth Haug & Henriksen* (1969) Streptomycin resistance transformation studies have revealed particular close relations between *M nonliquefaciens M bovis* and the serum liquefying nonhaemolytic moraxellae *Bovre* (1965a b)

Differentiation between the genera *Neisseria* and *Moraxella* may sometimes be difficult In that case it might be useful to apply *Henriksen s* (1952) conclusion that the only significant difference between the two genera is that one consists solely of cocci and the other primarily of rods *Murray & Truant* (1954) find that the physiological similarity between the two genera may be misleading but point out that morphologically *Moraxella* is distinctly different from *Neisseria* in that the Gram negative cocci divide in two planes whereas *Moraxella* constantly divide in one plane Genetic transformation studies and DNA base composition determinations have shown important taxonomic relations between *Moraxella* and *Neisseria* (*Bovre* 1967a b)

As regards cell morphology the strains of *M bovis* investigated by the author correspond to the descriptions given in literature and had the traits characteristic of the genus *Moraxella*

It was characteristic that in primary culture the colonies with a few exceptions were of the rough type described In subculture rapid dissociation to smooth colonies was observed It appears that the rough form is difficult to maintain, but the dissociation is reversible Previous investigations have shown that it is the smooth forms which occur on primary isolation *Barner* (1952) and *Jackson* (1953) *Jackson* (1953) considers the rough form avirulent The results of the present investigations are not in agreement with the above findings

All the strains examined by the author were catalase negative In their definition of the genus *Moraxella Baumann et al* (1968) assert that bacteria belonging to that genus are catalase positive *Pugh et al* (1966) examined 101 strains of *M bovis* and found 53 catalase positive and 48 catalase negative strains Hence *Moraxella bovis* seems to be variable as regards that criterium Variability as regards the catalase test should therefore be included in future definitions of the genus *Moraxella* The reaction in litmus milk—development of three zones—has been described previously In these descriptions there were variations in the degree of prior coagulation of casein In the present experiment, however, no actual prior coagulation of casein was observed

CONCLUSION

In the examination of swabs from cases of infectious keratoconjunctivitis certain criteria have proved to be particularly valuable for the identification of *M bovis* The haemolytic flat and rough colonies are typical but the bacteria may also appear in a smooth phase, in which case they may easily be mistaken for other haemolytic types of colonies The cell morphology described with the typical diplo forms, is a valuable criterium Among the biochemical reactions the positive oxidase reaction and the proteolytic properties of the organism are of greatest diagnostic importance especially the typical reaction in litmus milk should be emphasized *M bovis* does not attack carbohydrates and does not grow on MacConkey agar

Animal no	1		2		3		4		5		6		7		8		9		10		11	
Serum injected (6 ml)	Ragpthgl		Ragpthgl		RaFread		Ragpthgl		RaFread		Ragpthgl		RaFread		Ragpthgl		RaFread		Ragpthgl		RaFread	
Antigen injected (in mg)	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl	Thgl	Rggl

Fig 1 Experimental design Each animal is schematically drawn and approximate localization of the reaction sites in the skin is marked by circles The numbers in the circles show the amount of protein injected in mg S sites injected *with saline and N untreated sites Ragpthgl* = rabbit antiserum to guinea pig thyroglobulin RaFreadj = rabbit antiserum to Freund's complete adjuvant Thgl = guinea pig thyroglobulin Rggl = rabbit γ globulin

gestions we have provoked a direct passive cutaneous *Arthus reaction* in guinea pigs with thyroglobulin as antigen using the same anti thyroglobulin serum pool as previously utilized in the study of thyroiditis (*Kåresen & Godal* 1969a, b) (*Kåresen* 1970)

MATERIALS AND METHODS

tigen Two different batches of guinea pig ...roglobulin were used One was prepared by $(NH_4)_2SO_4$ fractionation (*Godal & Kåresen* 1967) the other was a highly purified product prepared by Sephadex G 200 and gel electrophoresis of $(NH_4)_2SO_4$ fractionated thyroglobulin (*Kåresen & Godal* 1969a) As a control for unspecific reactions rabbit γ globulin purchased from Hyland Laboratories was used

Antiserum For the production of rabbit antiserum to guinea pig thyroglobulin the highly purified thyroglobulin was used This antiserum contained 703 μg antibody N/ml it had a precipitating titre in gel of 1/100 a haemagglutinating titre against guinea pig thyroglobulin coated red cells of 1/100 000 and fixed complement in dilutions up to 1/300 It also contained trace amounts of antibodies to guinea pig serum which were absorbed out As a control serum from a rabbit injected with Freund's complete adjuvant was used Immunization absorption and characterization of antiserum as well as the production of fluorescein—isothiocyanate conjugated guinea pig antiserum to rabbit γ globulin have been described (*Kåresen & Godal* 1969a b)

Animals Eleven random bred white female guinea pigs strain Lon Sff-e weighing between 200 300 g were obtained from the National Institute of Public Health Oslo They were injected intravenously with 4 ml of either anti thyroglobulin or anti Freund's adjuvant antiserum. *Hours* after their backs were shaved with an electric clipper and the cutanous reaction sites were marked with black ink Then either guinea pig thyroglobulin rabbit γ globulin or 0 9 per cent saline were injected intradermally as indicated in Fig 1 The doses of protein varied from 0 001 to 0 8 mg in 0 ml 0 9 per cent saline In animal No 1 3 thyroglobulin of the highly purified type was used Ten random bred rabbits weighing 2000 3000 g were used for the production of antiserum

Histological techniques Six hours after the injection animal No 4 11 were either anaesthetized

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right side hemithyroidectomy was performed and skin biopsies taken as indicated in Fig 1 Twenty four hours after the injection all animals were killed by either the left thyroid lobes removed and skin biopsies taken as indicated in Fig 1

The skin biopsies from animals No 4–11 were cut in three pieces with a razor blade One piece was fixed in ethanol/formalin = 9/1 for Dominici (*Litt* 1963) and haematoxylin-eosin staining the second in cold ethanol for fluorescence microscopy (*Sainte Marie* 1962) and the third in Karnovsky's fixative (*Karnovsky* 1965) for the staining of mast cells The other skin biopsies and the thyroid lobes were cut in two and fixed in either ethanol/formalin = 9/1 for Dominici and hematoxylin/eosin staining or cold ethanol for fluorescence microscopy The tissues were embedded in paraffin and cut in approximately 6 μ thick sections The methods of preparation and microscopy of fluorescence microscopic sections have been described (*Karesen* & *Godal* 1969b)

Counting Eosinophil and neutrophil granulocytes were counted in one Dominici stained section from the skin and one from the thyroid in each animal The sections were from biopsies taken 24 hours after the injection The microscope was fitted with a 100 × objective and an 8 × ocular with a quadratic frame of known size The granulocytes in as many adjacent full frames as to exceed 500 cells in total were counted Mast cells were counted in 6 different sections from skin biopsies taken from normal untreated areas of 3 animals injected with anti Freund's adjuvant serum (Fig 1) using a 40 × objective and the same ocular as for the granulocytes Values for the upper dermis were obtained by counting all mast cells in 10 adjacent ocular frames in a row parallel to and in contact with the epidermis Values f r the lower dermis were obtained by counting all mast cells in the same number of frames in a row parallel to and in contact with the panniculus carnosus

RESULTS

Animals Receiving Antiserum to Thyroglobulin Intravenously

The thyroids All animals which were given anti thyroglobulin serum intravenously had infiltrates of granulocytes in their thyroids both 6 and 24 hours after the injection (Fig 2) Although the infiltrates were generally less extensive 6 hours after the injection there was no difference in the morphology of the infiltrates after 6 and 24 hours However the more thyroglobulin given intradermally, the less heavy were the infiltrates in the thyroid The eosinophil granulocyte was the dominating cell in all animals (Fig 4) Counting however revealed that there was a considerable but not systematic variation in the proportion of eosinophils to neutrophils from animal to animal—the percentage of eosinophils varying between 36 and 99 per cent of the granulocytes (Table 1)

The fluorescence microscopic observations were identical with those of a previous study (*Karesen* & *Godal* 1969b) Six hours f

TABLE 1 *Per cent of Eosinophil and Neutrophil Granulocytes in the Thyroid and the Skin of Guinea Pigs injected Intravenously with 4 ml Rabbit Anti Thyroglobulin Serum and Intradermally with* [illegible] *Doses of Guinea Pig Thyroglobulin*

Animal no	Thyroglobulin injected intradermally (In mg)	Percent of eosinophil and neutrophil granulocytes in relation to total granulocyte count			
		Skin		Thyroid	
		Neutr	Eosin	Neutr	Eosin
1	0 01	96	4		
	0 1	97	3	0	90
2	0 1	99	1		
	0 01	94	6	44	56
4	0 2	97	3	11	89
6	0 4	64	36	1	99
8	0 6	85	15	40	60
10	0 8	87	13	11	89

Animals Receiving Antiserum to Freund's Adjuvant Intravenously

The thyroids None of the animals had signs of inflammation in their thyroids, and green fluorescent material was only seen in vessel lumina

Skin sites injected with thyroglobulin No macroscopic reactions were noted

Microscopically no sign of inflammation were found at sites injected with doses up to 0 2 mg At higher doses an inflammatory reaction was seen with a morphology similar to that found in sites from animals receiving anti thyroglobulin serum intravenously and thyroglobulin subcutaneously The degree of reaction was however clearly less

No green fluorescence was found outside vessel lumina

Skin sites injected with rabbit γ globulin Observations similar to those in animals receiving anti thyroglobulin serum intravenously and rabbit γ globulin subcutaneously were made

Skin sites injected with saline and uninjected sites No macroscopic or microscopic inflammatory changes were noted in the lower dermis In the upper dermis scattered eosinophils were found

When mast cells in the upper dermis were counted a mean value of 82 cells/mm² was found In the lower dermis there were only 8 cells/mm²

DISCUSSION

The results demonstrate that the reaction between guinea pig thyroglobulin and rabbit antibodies to thyroglobulin gives rise to an inflammatory reaction of different morphology in the thyroid and in the skin of the same guinea pig In the thyroid the eosinophil granulocyte is the dominating inflammatory cell, while neutrophil granulocytes and mononuclear cells prevail in the skin

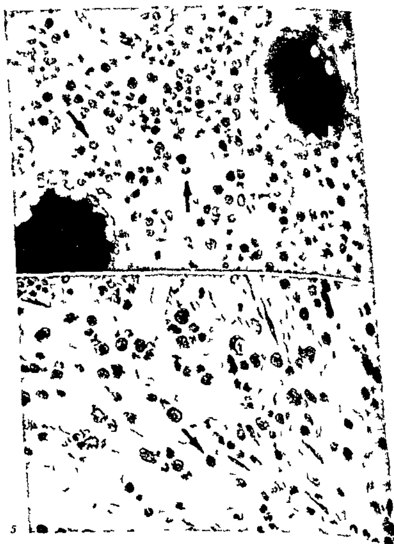
The reaction in the skin is similar to the results obtained when passive direct Arthus reaction is provoked in other species and with a variety of antigens and their corresponding antisera (*Cochrane* 1965) Lately *Nakamura* & *Weigle* (1969) have made similar observations when direct active Arthus reaction was provoked in rabbit using rabbit thyroglobulin as antigen This similarity in morphology with the Arthus reaction provoked by other antigens seems to justify the rejection of the suggestion that the high carbohydrate content of thyroglobulin could be an explanation of the thyroid eosinophilia (*Karesen* & *Godal* 1969a) It also seems to rule out the possibility that a special class of antibodies should be directly involved in eosinotaxis in the present experimental model As the antiserum provoking the inflammation in both the thyroid and the skin was injected intravenously one would expect that both organs were exposed to the antibodies in a similar fashion. The possibility that difference in perfusion rates the structure of vessel walls or other factors allow antibodies involved in eosinotaxis to be more selectively accumulated in the thyroid must be considered However this does not seem to be very likely especially in the light of the recent report by *Tolo* & *Fevang* (1969) indicating that blood eosinophilia in humans hypersensitive to penicillin is specifically associated with skin sensitizing antibodies

The suggestion of *Cohen* & *Sapp* (1967) that the number of antigen antibody complexes formed may be of significance for the development of eosinophilia may gain some support from this study as the skin sites in

Fig 4 Same section as in Fig 3 The infiltrate consists mainly of reddish stained eosinophil granulocytes A few neutrophil granulocytes (arrows) and some mononuclear cells are also seen Dominici staining × 430

Fig 5 Same section as in Fig 3 The infiltrate consists mainly of mononuclear cells and neutrophil granulocytes A single eosinophil granulocyte can be seen (arrow Dominici staining × 430

Fig 6 Skin site injected with 0 6 mg thyroglobulin from an animal given antiserum to thyroglobulin intravenously The biopsy was taken 4 hours after the injection the section was incubated with fluorescein conjugated guinea pig antibody rabbit γ globulin for ½ hour Accumulations of rabbit γ globulin are seen in a vessel wall and in inflammatory cells × 430


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A PARAFFIN EMBEDDING METHOD IN KIDNEY IMMUNOFLUORESCENT STUDIES

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Immunofluorescence microscopy was carried out on kidney biopsies fixed in cold ethanol embedded in paraffin and sectioned by a standard microtome Immunoglobulins could be demonstrated with glomerular localization in cases of kidney diseases in a pattern similar to that described by others who have investigated frozen specimens β1A/β1C complement was shown to be preserved less satisfactorily by the method The advantages of the method and precautions to be taken are discussed

The fluorescent antibody method for demonstration of tissue bound antigens and antibodies was introduced by *Coons and co workers* (2 3 4 5) The kidneys have been investigated by this method by many workers who have described the presence of immuno globulins in the glomerular basal membrane of kidneys from patients with various types of renal disease suggested to have an immuno logical basis (7) Most workers have investigated human renal tissue obtained by biopsy and they have all used a freezing technique for fixation and cutting of the tissue Chemical fixation has been avoided because antigen and antibody activity might be inactivated by conventional histological methods The routine histological examination must still be considered the most important Many pathologists are however, not satisfied with a freezing method for routine histology but want a paraffin embedding method Most clinicians avoid drawing more than one biopsy and if this is divided into two portions one often contains only medullary tissue without glomeruli and the other cortex substance with glomeruli which is obviously undesirable If all of the kidney biopsy could be frozen and used for immuno histochemical procedures as well as for routine histological examination there would be no problem

To solve the problem the author has used a paraffin-embedding technique for immunofluorescence studies on renal tissue This fixation method has been described by Sainte Marie who demonstrated that antibodies in rabbit lymph nodes are preserved better by this technique than by the classical technique of *Coons* (10) A few workers have used this paraffin technique for fixing synovial tissue for immunofluorescent studies and have found it superior to freezing methods (6)

MATERIAL AND METHODS

Renal biopsies were performed at Aalborg Centr Hospital on clinical indication When the tissue was obtained it was dropped into 95 per cent 4° C precooled ethanol The glass container was returned to the refrigerator and carefully stoppered It was left overnight at 4° C Next

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morning it was dehydrated with 99 per cent 4 C ethanol for 3 hours The next step was clearing in 4 C cold xylol through two consecutive baths for 3 hours in all While still containing the tissue the last bath was allowed to rise to room temperature (The specimen may be stored for 1–2 days either in absolute alcohol or in xylol at 4 C) The specimens were now placed in a paraffin oven at 58 C overnight On the third day the block was sectioned in an ordinary microtome Blocks were stored in the refrigerator for later use The section was flotated on water at a temperature of less than 40 C After a few seconds the section was picked up with a glass slide Adhesives such as Mayer s egg albumin were not ordinarily necessary but depended on the cleanliness of the glass slide At the beginning we used glass slides not specially cleaned and most sections fell off under the deparaffinization We now use glass slides degreased with chrome sulphuric acid and the section stays in place under the subsequent procedure

The deparaffinization goes on as shown in Table 1 It soon became obvious that pure paraffin was necessary If paraffin beewax was used the time for each step had to be much longer and the deparaffinization was often unsatisfactory After gently washing in phosphate buffered saline the section was ready for exposure to the immunological reagent

TABLE 1 *The Fixation and Deparaffinization Procedure is Listed in Detail*

1st day

Draw biopsy
Drop into ethanol 95 per cent 4 C
Leave in refrigerator overnight

2nd day

Place in ethanol 99 per cent 4 C for 3 hours
Pass through two xylol baths 4 C for 3 hours
Let last bath rise to room temperature
Place in paraffin oven 58 C overnight

3rd day

Section (Block may be stored in refrigerator for months before sectioning)
Deparaffinize
Incubate with immunological reagent

Deparaffinization

Section on degreased glass slides

c 60 sec xylol 4 C two changes
c 60 sec ethanol 99 per cent 4 C two changes
c 10 sec ethanol 96 per cent 4 C one bath
c 10 sec ethanol 70 per cent 4 C one bath
c 60 sec buffered saline 3–4 changes

Air-drying

The immunological reagents used have been bought commercially* Routinely a fluorescence conjugated rabbit antihuman immunoglobulin reagent was used for one section and a sandwich technique for detecting complement for another This latter includes horse antihuman b1C-b1A globulin as a first layer and a fluorescence conjugated rabbit anti horse immunoglobulin as a second layer In some cases IgG IgM IgA globulins fibrinogen and albumin were detected in a similar sandwich technique using rabbit anti human proteins as first reagent and FITC conjugated horse anti rabbit immuno-globulins as the second The incubation time was 30 minutes The slides were then washed in Coons buffered saline for 30 minutes The reagents were tested in different dilutions and the optimal was a ten time dilution of the fluorescence conjugated and a two-time dilution of the non conjugated reagent After incubation the slides were washed for one hour and air dried for a few minutes and covered with a coverglass over a drop of buffered glycerol A number of control sections were examined using unlabelled antisera and conjugated sera against rabbit immunoglobulins Other control sections were treated with a non FITC conjugated antiserum prior to incubation with labelled antibody The sections were studied in a Leitz Orthoplan microscope having a 200 Watts HBO high tension mercury vapor bulb as a light source A dark field condenser was employed and a bluelight filter was preferred in most instances Photographs were taken with a Leitz Orthomat automatic camera using Kodachrom and Ilford films

Sections for routine histological examination were stained with haematoxylin eosin PAS method and van Gieson staining

RESULTS

19 cases of glomerulonephritis of different types and stages were examined Immunoglobulins especially IgG globulin and complement were shown within the basal membrane in most cases In cases of acute glomerulonephritis the globulins could be seen in a slightly granular fashion along the basal membrane in most of the glomeruli, (Fig 1) but there was usually minimal fluorescent staining of the mesangium One case revealed linear (smooth) deposits along the basal membranes Frequently protein

* Centraal Laboratorium van de Bloedtransfusiedienst van Het Nederlandsche Roede Kruis and Nordic Pharmaceuticals and Diagnostics.

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CHARACTERIZATION OF THE ACID PHOSPHATASE ACTIVITY IN THE PLASMA MEMBRANE FRACTION FROM BABY HAMSTER KIDNEY CELLS (BHK 21)

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We have studied the acid phosphatases in the plasma membrane fraction from baby hamster kidney cells (BHK21) The plasma membrane acid phosphatases utilized various mono phosphates including mononucleotides and adenosine diphosphate but not adenosine triphosphate They also had phosphotransferase activity with glycerol as acceptor No requirement for magnesium was found The acid phosphatases from the plasma membrane and lysosomal fractions were almost completely inhibited by fluoride and tartrate ions and to a smaller extent by sulphate ions However the enzymes in the soluble fraction were much less sensitive to these inhibitors In Triton disc electrophoresis the acid phosphatase banding pattern with 2 glycerophosphate as substrate was found to be characteristic for the plasma membrane fraction This pattern clearly differed from the banding patterns obtained with the lysosomal and soluble fractions

Since the original studies by *de Duve et al* (1955) of the distribution of acid phosphatases (EC 3 1 3 2) in rat liver homogenates, the nature and subcellular localization of these enzymes have been extensively investigated Acid phosphatases have been thought to be located primarily in the lysosomes and to some extent in the microsomal and soluble fractions of many cells (*Goodlad & Mills* 1956 *Munro et al* 1964 *Nelson* 1966 *Cristofalo et al* 1967) Molecular heterogeneity has been shown by pH activity curves the effect of different inhibitors and electrophoresis (*Neil & Horner* 1964 *Lundin & Allison* 1966 *Nelson* 1966 *Cristofalo et al* 1967 *Wang* 1969) Acid phosphatase activity was detected by *Emmelot et al* (1964) and *Lansing et al* (1967) in isolated plasma membranes from normal rat liver cells with p nitrophenylphosphate as substrate and by *Molnar et al* (1969) in plasma membranes isolated from Ehrlich ascites carcinoma cells *Emmelot et al* (1964) found that the activity with 2 glycerophosphate (β glycerophosphate) was low They therefore considered that the plasma membrane acid p nitrophenylphosphatase was a distinct enzyme The acid phosphatase activity from nucleated cell membranes has not so far been further characterized Recently *Berry & Hochstein* (1969) reported the presence of an acid phosphatase activity in rabbit erythrocyte membranes which showed a high activity with various

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TABLE 1 *Fluoride and tartrate inhibition of BHK21 cell acid 2 glycerophosphatases*

Inhibitor		Per cent inhibition		
		Plasma membrane fraction	Lysosomal fraction	Soluble fraction
Fluoride	20 mM	100	100	71 4
Fluoride	2 mM	100	100	50 0
Tartrate	20 mM	93 3	93 2	51 0
Tartrate	2 mM	91 7	91 7	25 0

phate ions inhibited both the plasma membrane and lysosomal acid phosphatases but had less effect on the soluble enzymes

Substrate Spectrum

Table 2 shows the plasma membrane acid phosphatase activities with different substrates The plasma membrane acid phosphatases had the highest activity with p nitrophenyl phosphate 2 glycerophosphate or glucose 6 phosphate as substrate Activity was also high with the different nucleotide monophosphates and with adenosine diphosphate but not with

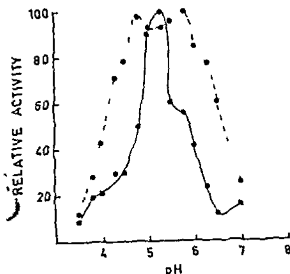


Fig 3 The effect of pH on hydrolysis of 2 glycerophosphate by BHK21 soluble fraction The results are expressed in per cent of maximal activity in the absence (●——●) and in the presence (●- - -●) of 10 mM magnesium chloride Maximal activities 0 22 and 0 72 μmoles inorganic phosphorus released per mg protein per hour at 37 respectively The samples were incubated in 0 1 M acetate buffers at 37 for 60 min.

adenosine triphosphate All these activities were efficiently inhibited by fluoride ions

Phosphotransferase Activity

The plasma membranes had a marked phosphotransferase activity at pH 5 with glycerol as acceptor (Table 3) The release of p nitrophenol was not much affected by the presence of glycerol The activity was inhibited by fluoride

Triton Disc Electrophoresis

Fig 5 shows the polyacrylamide gels of the plasma membrane and lysosomal fractions stained for 2 glycerophosphatase activity The same gels are shown schematically in Fig 6

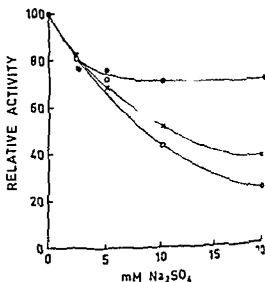


Fig 4 The effect of sulphate ions on hydrolysis of 2 glycerophosphate by BHK21 plasma membranes (×) lysosomal fraction (0) and soluble fraction (●) The samples were incubated in 0 1 M acetate buffer pH 5 0 at 37 for 60 min.

TABLE 2 *Inorganic Phosphate (P) Released from Various Substrates by BHK21 Plasma Membranes in the Absence and Presence of 10 mM Sodium Fluoride*

Substrates	Additions	µmoles P	Per cent inhibition
2 glycerophosphate	None	1 40	
	NaF	0	100
p nitrophenylphosphate	None	4 00	
	NaF	0 47	89 2
Glucose 6 phosphate	None	1 63	
	NaF	0 10	93 9
Adenosine triphosphate	None	0 038	
	NaF	0	100
Adenosine diphosphate	None	0 63	
	NaF	0	100
Adenosine monophosphate	None	0 38	
	NaF	0	100
Cytosine monopho phate	None	0 61	
	NaF	0	100
Uridine monophosphate	None	0 80	
	NaF	0	100
Guanosine monopho phate	None	0 35	
	NaF	0	100

TABLE 3 *The Transfer of Inorganic Phosphate (P) from p nitrophenylphosphate to Glycerol by BHK21 Plasma Membrane Fraction*

Additions	µmoles of free P	Per cent inhibition (P)	µmoles p nitro phenol relased	Per cent inhibition (p nitro phenol)
None	4 32		4 16	
NaF 10 mM	0 46	89 4	0 37	91 1
Glycerol (15%)	2 00		4 76	
Glycerol (15%) + NaF (10 mM)	0 08	96 0	0 59	87 6

Some acid phosphatase did not penetrate into the gels Four bands were obtained from the plasma membranes from the cathodal to the anodal end of the gel named A B C and D Bands A and C were the strongest in the plasma membrane fraction With fluoride ions in the incubation medium these bands became much weaker but were still visible From the lysosomal fraction two bands were obtained corresponding to A and B of the plasma membranes Band B was much stronger than band A Band B was very weak when the gel was incubated with the sub strate medium in the presence of fluoride ions If 2 glycerophosphate was omitted from the incubation medium band B was still weakly stained (in the plasma membrane gel) This could probably be explained by the presence of a large number of SH groups in this protein reacting with lead ions If the soluble fraction was stained for 2 glycerophosphatase activity a complex pattern was obtained with at least four bands One of these corresponded to band B but the rest were more anodal than band D Thus the major lysosomal acid pho phatase (band B) was found probably as a contaminant in both the plasma membrane and soluble fractions

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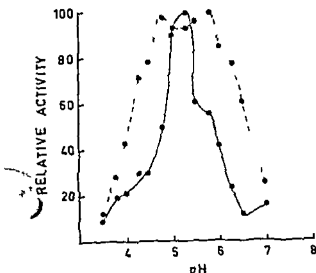


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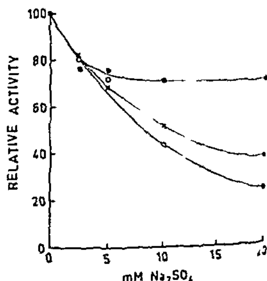


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CHARACTERIZATION OF THE Lp(a) LIPOPROTEIN IN HUMAN PLASMA

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The Lp(a) lipoprotein from human plasma of Lp(a+) phenotype was isolated by preparative ultracentrifugation and Sepharose 2B gel filtration The lipoprotein was found in the plasma fraction with a density of 1 05–1 12 g/ml and had pre-beta mobility in agarose electrophoresis The molecular weight was estimated by gel filtration to be 4 8 10^6 and by electron microscopy to be 5 6 10^6 Electron micrographs of negatively stained Lp(a) lipoprotein showed a spherical structure Electrofocusing gave an iso-electric point of 4 9 for this lipoprotein and of 5 5 for the low density lipoproteins (LDL) In amino acid composition the Lp(a) lipoprotein differed from both LDL and high density lipoproteins (HDL) whereas in lipid composition it was similar to LDL Both Lp(a) lipoprotein and LDL contained small amounts of sialolactosylceramide The Lp(a) lipoprotein contained about five times more protein bound sialic acid than did LDL The total lipid content of the Lp(a) lipoprotein was 1 8 mg lipid per mg protein this is lower than the value for LDL but higher than that for HDL

Two independent immunological systems that determine the synthesis of inherited variants of the human plasma beta or low density lipoproteins (LDL) have been detected The first the Ag system has a complexity approaching that of the Gm system of IgG globulins its various types are recognized by the use of human iso-immune precipitating antisera (*Giblett* 1969) The second the Lp system is less complex Its two phenotypes Lp(a+) and Lp(a−) can be distinguished by immunodiffusion with heteroprecipitins (*Berg* 1963) and by polyacrylamide gel electrophoresis (*Garoff et al* 1970) In Lp(a+) individuals the Lp(a+) activity is found in a plasma fraction with a higher density 1 050–1 125 g/ml than LDL (*Wiegandt et al* 1968 *Schult et al* 1968) *Utermann & Wiegandt* (1969) isolated the Lp(a) lipoprotein and showed that it contained less lipid than the bulk of the LDL lipoproteins but shared antigenic determinants with them The frequency of Lp(a−) individuals in Caucasian populations is about 0 7 (*Berg* 1968) and they seem to have no or very low levels of Lp(a) lipoprotein

We have isolated Lp(a) lipoprotein by preparative ultracentrifugation and Sepharose 2B gel filtration and found that it differs from

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Abbreviations LDL low density lipoproteins (1 019 1 050 g/ml) HDL high density lipoprotein (1 063 1 ?0 g/ml) HDL (1 063–1 125 g/ml) TLC thin layer chromatography

24 *Nordlie R C* & *Arion W J* Glucose-6 phosphatase In Colowick S P & Kaplan N O (Ed) Methods in enzymology vol 9 Academic Press Inc New York 1966 p 619–625

25 *Post R L Merritt C R Kinsolving C R* & *Albright C D* Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte J biol Chem *235* 1796–1802 1960

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Abbreviation LDL low density lipoprotein (1 019–1 050 g/ml) HDL high density lipoprotein (1 063–1 210 g/ml) HDL (1 063–1 125 g/ml) TLC thin layer chromatography

all the main plasma lipoproteins The lipoprotein had pre beta mobility in agarose electrophoresis a molecular weight of about 5 million, and a high sialic acid content In amino acid composition it differed from both LDL and high density lipoproteins (HDL) whereas in lipid composition it resembled LDL

MATERIAL AND METHODS

Isolation of Lp(a) Lipoprotein

Immunological and electrophoretic Lp typing were performed as described elsewhere (*Garoff et al* 1970) The density fraction 1 05–1 12 g/ml was isolated by ultracentrifugation from plasma of Lp(a+) individuals as described by *Havel et al* (1955) It was then fractionated on a Sepharose 2B column (2 5 × 95 cm) in 0 1 M sodium chloride 0 05 M sodium phosphate and 0 05 per cent disodium ethylenediaminetetraacetate pH 7 5 The void volume (160 ml) of the column was determined with blue dextran and the total volume (490 ml) with radioactive iodide The Lp(a) lipoprotein eluted before LDL which was followed by HDL Lp(a) lipoprotein and LDL were not completely separated in the first gel filtration and refractionation was necessary The purity of the Lp(a) lipoprotein preparation was tested by 1) disc electrophoresis in 3 75 per cent polyacrylamide gels at pH 8 4 (*Garoff et al* 1970) 2) double diffusion in agar gel and 3) immuno-electrophoresis at pH 8 6 in 1 per cent agarose gels LDL (density 1 019 1 050 g/ml) was isolated as described previously from Lp(a+) plasma (*Havel et al* 1955 *Simons & Helenius* 1969) The LDL preparations did not contain Lp(a) lipoprotein

E[illegible] ofocusing

We used electrofocusing in a LKB Electrofocusing 1 column to determine the iso-electric point of Lp(a) lipoprotein (*Vesterberg & Svensson* 1966) The density fraction 1 05–1 12 g/ml from Lp(a+) plasma was used for these studies The run was performed in a pH gradient from 4–6 at 500 V for 72 hours at 15 C Lubrol W (0 1 per cent) was included in the ampholyte solution (LKB) to prevent precipitation The absorbance at 280 nm of the eluted fractions was measured and the Lp(a) lipoprotein and LDL were localized by immunodiffusion with specific antisera

Estimation of Molecular Weight

The Sepharose 2B column was calibrated with polio virus bacteriophage Φ×174 and LDL The distribution coefficient K[illegible] was calculated for each marker and for Lp(a) lipoprotein as described by *Laurent & Killander* (1964) From these values the apparent molecular weight was determined

Electron Microscopy

One drop of the lipoprotein preparation to be examined was placed on a carbon coated 400 mesh grid The grid was then carefully washed with a 2 per cent solution of sodium tungstosilicate pH 6 5 in order to avoid salt crystals in the dried preparation After washing the excess fluid was withdrawn by touching the edge of the grid with a filter paper The grid was then immediately transferred to the vacuum of a Philips EM 200 The specimens were examined at a magnification of 46600 using double condensed illumination and an operating voltage of 80 kilovolts The magnification was calibrated with carbon gratings The lipoprotein diameters were obtained from the electron micrographs by edge-to-edge measurement of the electron transparent images

Chemical Analysis

Protein was determined by the method of *Lowry et al* (1951) with bovine serum albumin as standard using 0 1 per cent sodium dodecyl sulphate in the reaction mixture Lipoprotein solutions were lyophilized and their lipids extracted immediately with chloroform-methanol as described elsewhere (*Renkonen et al* 1970) The total lipids were partitioned in the Folch system in which gangliosides are separated from all other lipids The la[illegible] fraction was treated with a chelating resin which converts the acidic lipids to sodium salts (*Carter & Weber* 1966) After this the neutral lipids glycolipids and phospholipids were separated from each other on silicic acid columns (*Rouser et al* 1[illegible]) The ganglioside fraction was dialysed lyophilized and the gangliosides were extracted from the [illegible] residue with chloroform methanol (2 1) (v/v)

Aliquots of the neutral lipid fractions were weighed The glycolipid fractions were methanolyzed the methyl glycosides were purified converted to trimethylsilyl ethers and subjected to gas liquid chromatography on *SE-30* (*Vance & Sweeley* 1967) The amounts of glucose and galactose were obtained by using mannitol as internal standard in this procedure To obtain the glycolipid figures the amount of glucose was multiplied by 4 The phospholipid and ganglioside fractions were assayed by measuring phosphorus (*Fiske* [illegible] 1959) and sialic acid (*Warren* 1959) To obtain the lipid figures the amount of phosphorus was multiplied by 25 and that of sialic acid by 4

The neutral lipid fraction was further fractionated by thin layer chromatography (*TLC*) on silica gel G plates Double developments with hexane-ether (19 1) (v v) and hexane-ether-acetic

acid (50 50 1) (v/v/v) was used The individual components were analysed by the method of *Marsh & Weinstein* (1966)

The glycolipid fraction was studied by TLC essentially as described by *Vance & Sweeley* (1967) The plates were stained with diphenylamine reagent (*Bailey & Bourne* 1960)

The phospholipid classes were separated and estimated with standard techniques of two-dimensional TLC (*Rouser et al* 1967)

TLC of the ganglioside fractions was carried out on silica gel G plates with propanol – water (7 3) (v/v) (*Kuhn & Wiegandt* 1963) The lipids were stained with resorcinol spray (*Svennerholm* 1957)

After lipid extraction the protein residues of Lp(a) lipoprotein and of LDL were hydrolysed with 6 M HCl for 22 hours at 110 C (*Crestfield et al* 1963) Amino acid analysis was performed in the Beckman 120 C amino acid analyser Half cystine was determined as cysteic acid after performic acid oxidation Tryptophan was determined after $Ba(OH)_2$ hydrolysis (*Noltman et al* 1962) All values are expressed as moles of amino acid per 1 000 moles of all amino acid residues recovered Protein bound sialic acid was determined by the resorcinol method of *Svennerholm* (1958) as modified by *Miettinen & Takki Luukkainen* (1959)

Antisera

Rabbit antisera to human serum HDL and LDL were purchased from Behringwerke AG The anti Lp(a) serum has been described elsewhere (*Garoff et al* 1970)

RESULTS

Electrophoretic Studies

Polyacrylamide gel electrophoresis in 3 75 per cent gels revealed three bands in the 1 05–1 12 g/ml fraction from Lp(a+) plasma a rapidly migrating HDL band a slowly migrating LDL band and a very slow band containing the Lp(a) lipoprotein (Fig 1) This agrees with previous findings (*Wiegandt et al* 1968 *Garoff et al* 1970) This plasma fraction gave a bimodal precipitin line with anti LDL analysed in agarose immunoelectrophoresis at pH 8 6 The Lp(a) lipoprotein formed the anodal mode and LDL the cathodal mode of this precipitin line (Fig 2) The Lp(a) lipoprotein thus had pre beta mobility in this system The 1 05–1 12 g/ml fraction from Lp(a–) plasma did not con

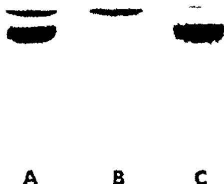


Fig 1 Polyacrylamide gel electrophoresis of A fraction of density 1 05–1 12 g/ml from Lp(+) plasma (HDL band not shown) B purified Lp(a) lipoprotein and C LDL (1 019–1 050 g/ml) Prestained with Sudan black Anode at the bottom

tain a component corresponding to the Lp(a) lipoprotein It is interesting that the electrophoretic variant described by *Seegers et al* (1965) is very similar to the Lp(a) lipoprotein This variant may belong to the Lp system and warrants further study

Iso electric Point

Electrofocusing experiments gave an iso-electric point of 4 9 for the Lp(a) lipoprotein This is lower than the value we got for LDL 5 5 *Aurault Jarner* (1959) obtained an iso-electric point of 5 7 for LDL using electrophoresis

Purity Criteria for the Isolated Lp(a) Lipoprotein

The isolated Lp(a) lipoprotein preparations only gave one band in polyacrylamide gel electrophoresis (Fig 1) The samples applied to these gels were prestained with Sudan black B Protein staining with Coomassie blue (*Chrambach et al* 1967) did not reveal any other bands Only one precipitin line was obtained in both immunodiffusion and immuno-electrophoresis against anti human serum,

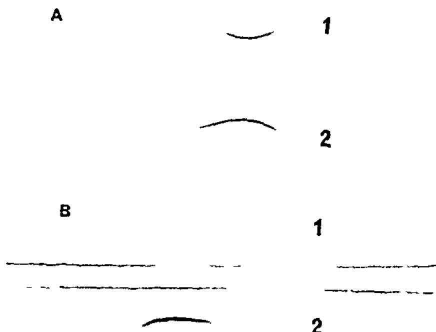


Fig 2 Immuno-electrophoresis in agarose gel. In the antigen wells 1 fraction of density 1.05–1.12 g/ml from Lp(a−) plasma and 2 same fraction from Lp(a+) plasma. Antisera in A anti LDL and in B Lp(a). Anode to the left. Stain Oil Red O

anti LDL and anti Lp(a) serum. These Lp(a) lipoprotein preparations were used for electron microscopy and for the chemical analyses. It should be pointed out that the Lp(a) lipoprotein became quite labile in the later stages of purification and showed a troublesome tendency to aggregate.

Estimation of Molecular Weight by Gel Filtration

The apparent molecular weight of the Lp(a) lipoprotein was estimated by plotting K_{av} against the logarithm of the molecular weight (Fig 3). For this plot we assumed that the molecular weight of polio virus is 6.8×10^6 (*Schwerdt* 1957), of bacteriophage $\Phi X 174$ is 6.2×10^6 (*Sinsheimer* 1959) and of LDL is 3.5×10^6 (determined by gel filtration by *Margolis* 1967). Using these values the apparent molecular weight of the Lp(a) lipoprotein was found to be 4.8×10^6

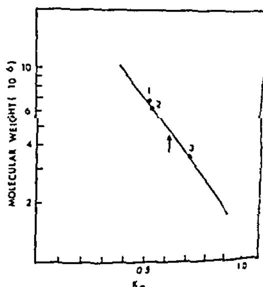


Fig 3 Molecular weight determination of the Lp(a) lipoprotein with Sepharose 2B gel filtration. The following markers were used 1 polio virus, 2 bacteriophage ΦX 174 and 3 LDL. The arrow indicates K_{av} of the Lp(a) lipoprotein.

Electron Microscopy

Electron micrographs of Lp(a) lipoprotein and of LDL are shown in Fig 4. These were obtained after negative staining with sodium tungstosilicate. Both appear spherical (cf *Forte et al* 1968). The mean diameters of the Lp(a) lipoprotein and LDL particles were found to be 255 Å and 225 Å respectively. The molecular weights were calculated* using these diameters, a density of 1.07 g/ml for Lp(a) lipoprotein (*Schultz et al* 1968) and a density of 1.035 g/ml for LDL. The molecular weight of Lp(a) lipoprotein was 5.6×10^6 and of LDL 3.6×10^6. These figures are in fair agreement with the values determined by gel filtration.

Chemical Analysis

The ratio of total lipid to protein was 1.8 mg lipid per mg protein in Lp(a) lipoprotein and 3.75 mg lipid per mg protein in LDL. The value for LDL agrees with the ratio obtained by *Skipski et al* (1967). HDL has a lipid to protein ratio of 0.9 (*Skipski et al* 1967).

The lipid compositions of the Lp(a) lipoprotein, LDL and HDL are shown in Table 1. In lipid composition the Lp(a) lipoprotein resembled LDL to a remarkable extent. The ratio of neutral lipids to phospholipids and the distribution of the major phospholipids is similar in the two lipoproteins. In both respects they are clearly different from HDL. Both Lp(a) lipoprotein and LDL contained gangliosides. The principal component was identified as sialolactosylceramide by TLC. As far as we know this is the first time that haematosides have been demonstrated in human plasma lipoproteins.

TLC of the glycolipid fractions revealed in LDL and Lp(a) lipoprotein components which moved like ceramide monosaccharides and ceramide disaccharides. Gas liquid chromatographic analysis of the carbohydrates revealed consistently 2–3 times more glucose than galactose in the glycolipid fractions. *Vance & Sweeley* (1967) have reported similar figures in the case of ceramide mono- and disaccharides of whole plasma.

The amino acid compositions of the Lp(a) lipoprotein and LDL were similar in some respects but striking differences were also apparent, for example in half cystine content. The amino acid composition we obtained in the case of LDL is in good agreement with previously determined values (*Levy et al* 1967). The amino acid composition of the Lp(a) lipoprotein bears little resemblance to the reported amino acid composition of HDL (*Levy et al* 1967).

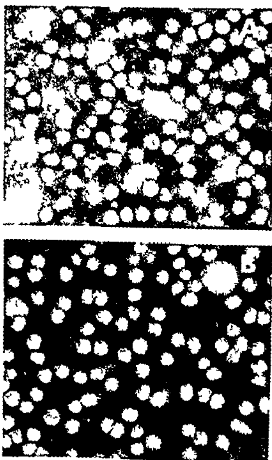


Fig 4 Electron micrograph of A Lp(a) lipoprotein and B LDL. Both negatively stained with sodium tungstosilicate. Magnification 156500 ×

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* Molecular weight was calculated from the expression $MW = \frac{4}{3}\pi r^3 \rho N$, where r = particle radius, ρ = average density and N = Avogadro's number.

TABLE 1 *Lipid Composition of Plasma Lipoproteins Per Cent of Total Lipid*

Lipid	Lp(a) lipoprotein Present work	Lp(a) lipoprotein Utermann & Wiegandt	LDL Present work	LDL Skipski et al	HDL Skipski et al.
Neutral lipids	77[e]	67	74[b]	72	58
Glycolipids	0.4[d]	nd	0.4[a]	0.2[h]	0.[illegible]
Phospholipids	23[c]	31	27[b]	28	42
Gangliosides	0.15[c]	nd	0.2[b]	nd	nd
Composition of neutral lipid					
Cholesterol esters	59[g]	29	56[f]	47	35
Cholesterol	14[g]	12	13[f]	12	7.4
Triglycerides	3[g]	3.9	[illegible][f]	6.2	4.6
Diglycerides	traces[g]	9.5	nil[f]	0.4	0.7
Monoglycerides	nil[g]	4.6	nil[f]	0.7	0.8
Free fatty acids	trace[b]	8.4	trace[f]	1.1	1.7
Composition of phospholipid					
Phosphatidylethanolamines	0.3[f]	nd	0.3[a]	0.6	1.4
Phosphatidylcholines	14[f]	nd	17[a]	18	31
Sphingomyelins	7.6[f]	nd	8.3[a]	7.2	6.1
Lysophosphatidylcholines	0.1[f]	nd	0.2[a]	0.8	0.8

nd = not determined a one sample one analysis b one sample two analyses c three samples three analyses d four samples four analyses e five samples five analyses f two samples two analyses g two samples four analyses h ceramide monohexosides only

TABLE 2 *Amino Acid Composition (Moles/1000 Moles) of Lp(a) Lipoprotein LDL and HDL*

Amino acid	Lp(a) lipoprotein[a]	LDL[a]	HDL[b]
Lysine	62.8	75.9	101.1
Histidine	21.3	23.0	17.1
Arginine	40.3	33.3	44.8
Aspartic Acid	98.9	106.7	81.4
Threonine	68.0	65.2	49.7
Serine	78.2	80.2	66.0
Glutamic Acid	117.8	122.0	173.6
Proline	54.1	39.7	41.0
Glycine	75.1	52.1	44.0
Alanine	82.6	61.4	86.4
Half Cystine	17.8	6.0	7.3
Valine	60.1	54.1	60.3
Methionine	15.8	17.1	9.0
Isoleucine	43.5	56.7	9.8
Leucine	84.9	118.6	124.5
Tyrosine	34.8	30.3	32.6
Phenylalanine	35.2	50.4	37.5
Tryptophan	8.7	6.4	9.8

a Mean of four different preparations
b Calculated from the data of *Levy et al* (1967)

The protein bound sialic acid content of the Lp(a) lipoprotein was found to be 30 µg/mg protein whereas the corresponding value for LDL was much lower 5.6 µg/mg protein. The high sialic acid content of the Lp(a) lipoprotein could explain its low iso-electric point its pre beta mobility in agarose electrophoresis and its retention on anion exchange columns (*Roelcke et al* 1968) Detailed carbohydrate analyses of these proteins are in progress

DISCUSSION

The Lp(a) lipoprotein is found in the same density fraction as HDL but these lipoproteins are otherwise very dissimilar Their amino acid and lipid compositions are different The molecular weight of the Lp(a) lipoprotein ($4.8 \cdot 10^6$ by gel filtration and $5.6 \cdot 10^6$ by electron microscopy) is much higher than the molecular weight of HDL or LDL This difference in size enabled us to

separate the Lp(a) lipoprotein from HDL and LDL by agarose gel filtration Its mobility (pre beta) in agarose electrophoresis also differs from that of HDL or LDL

The Lp(a) lipoprotein resembles LDL to some extent Firstly these lipoproteins share antigenic determinants Secondly their lipid compositions are quite similar Thirdly both appeared spherical in the electron micro scope Fourthly both Lp(a) lipoprotein and LDL are precipitated by dextran sulphate (our unpublished studies)

We do not know which part of the mole cule is involved in the antigenic determinants specific for the Lp(a) lipoprotein or those common to Lp(a) and LDL It seems likely that it is the protein part It will therefore be very interesting to compare the polypep tide chain compositions of these two lipo proteins We hope that the inherited varia tions in the beta lipoproteins will turn out to be as useful tools in elucidating the molecular architecture of these proteins as the Gm and Inv factors have been in clarifying the hetero-geneity and structure of the immunoglobu lins (*Natvig & Kunkel* 1968)

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immunized horses. Ten human sera containing AST active M components were studied for their capacity to neutralize the four bacterial haemolysins mentioned above. The results of this analysis are reported in the present paper.

MATERIAL AND METHODS

Human serum. Serum samples with M-components with AST activity were obtained from ten individuals and were stored at −16 to −20 C. The samples contained from 13 000 to 200 000 IU of AST per ml. The titres of samples withdrawn from time to time were found nearly unchanged for each of the patients for periods up to 11 years. Some characteristics were common for all ten sera: 1) The M-components belonged to the γG class of immunoglobulins. 2) The AST activity was confined to the M-components as readily demonstrated by the use of the method which combines haemolysis inhibition and immunoelectrophoresis (15). 3) The apparently monoclonal γG globulins were analogous to immune antibodies as the activity was restricted to their Fab and F(ab) fragments (16). 4) No titre changes were found after precipitation of lipoproteins by dextran (4), which is considered a criterion of specificity (7).

Control human sera. Human sera received for routine determination of AST content were used as controls. The range of titres were from <25 to 6 000 IU/ml. The titres were unchanged or insignificantly changed after dextran precipitation (4).

Human serum samples with a high concentration of unspecific streptolysin O inhibitors (8, 11), which were removable by dextran precipitation (2), were used for a few experiments.

Three sera from horses were investigated.

The antistreptolysin O serum used was Todd's standard serum containing 40 000 IU/ml.

The anti gas gangrene serum was a commercially available sample (Burroughs Wellcome Gas/Ser batch no. 10781). This preparation contained enzyme treated horse globulins.

The tetanus antitoxin was a globulin fraction prepared at this Institute from pepsin treated horse serum.

Rabbit red blood cells were only used on the day the blood was withdrawn by heart puncture. Blood of individual rabbits was shaken for 10 minutes to prevent clotting. Red blood cells were packed by centrifugation and washed 3 times with phosphate buffered saline pH 7.38. Cells were finally packed by centrifugation and a 5 per cent suspension of packed cells was employed. This indicator was always used in a volume giving a final concentration of 0.5 per cent red blood cells.

Streptolysin O. A broth filtrate from a culture of group A streptococci strain S 84 was prepared as described by *Kalbak* (13) and used without further purification. In order to ensure complete reduction, the streptolysin O was mixed with an equal volume of a neutral 0.067 M solution of cysteine 10 minutes prior to use.

Pneumolysin. A broth filtrate from a culture of *Dip. pneum.* type 0 B was prepared according to the procedure used for the streptolysin O preparation. Reduction of the pneumolysin thus obtained was carried out as mentioned for streptolysin O.

Tetanolysin. The tetanolysin used was a crude tetanus toxin which was obtained by filtration of cultures of *Cl. tetani* Albany strain 415 in a semi-synthetic medium (5). Toluene was used as a preservative during storage. The toluene was removed and the toxin was filtered through a membrane (Schleicher & Schüll no. 1121) before reduction with cysteine and before the experiments were carried out.

θ toxin. A commercially available lecithinase C (Sigma) was found to contain a sufficient amount of θ toxin for use in our experiments. The haemolytic activity of the θ toxin (= lecithinase C) was suppressed by the use of a phosphate buffer (14). A stock solution of 10 mg lecithinase C per ml was prepared in phosphate buffered saline containing 50 per cent glycerol. A dilution [illegible] (50 μg/ml) was prepared and reduced with cysteine prior to use.

CHU. One complete haemolytic unit (CHU) of the lysins was defined as the smallest volume which when diluted to 2 ml gave total haemolysis of 0.5 ml 2.5 per cent rabbit erythrocytes. The determination was carried out by adding red blood cells to tubes containing two-fold dilutions of the lysin. The mixtures were incubated for 45 [illegible] at 37 C and the degree of haemolysis read when intact cells had settled at room temperature. The volume containing one CHU of streptolysin O, pneumolysin, tetanolysin and θ toxin was determined with and without the [illegible] cysteine in order to follow possible changes during storage.

Antistreptolysin O. The determination of the number of international units of antistreptolysin O (AST) present in serum samples was carried out with minor modifications of *Kalbak*'s method (13).

The *titration of serum samples* against constant doses of the [illegible] lysins was carried out in a similar manner. The haemolysins were used in doses of [illegible] 6 CHU. As no standard sera were available except for AST, the titre of a serum against a particular haemolysin was expressed as the number of complete haemolytic units (CHU) which were neutralized by 1 ml of serum (CHU

TABLE 1 *Titration of Sera from Man and Horse with Oxygen Labile Bacterial Haemolysins*

	IU AST/ml	Anti haemolytic units/ml			
		Streptolysin O	Pneumolysin	Tetanolysin	θ toxin
Horse sera					
Anti strept	20 000	45 000	7 000	1 500	12 000
Anti welchii	2 000	4 000	3 300	1 300	15 000
Tetanus antitoxin	700	2 000	<128	256	512
Human sera					
1 M serum	200 000	450 000	1 000 000	<6	250
9 M sera	13 000–125 000	26 000–400 000	<128–128*	<13–<32	<50–50
8 sera	<12–225	<32–512	<64–256	n d	<26–100
12 sera	360–5 600	1 000–33 000	<128–512	<6–26	50–8 000

* One patient serum 1 024 n d = not done

The number of A HU/ml of the individual horse serum samples are listed for each of the haemolysins The values obtained for human sera are grouped except for the sample from one patient whose serum with an M component is extraordinarily potent in neutralizing streptolysin O and pneumolysin The control human sera include 8 with normal and 12 with elevated AST content

A 1 50 dilution of serum was generally used in a volume of 1 ml for the first tube of serial two-fold dilutions When antibody concentration was low a primary dilution of 1 10 was used whenever possible Sera with high titres were first diluted 1 1000 or 1 5000 and further dilutions were made from this in order to limit the number of dilution tubes to nine

RESULTS

CHU The CHU of the individual lysins showed minor day to day variation and was consequently determined for each set of experiments This variation was probably due to a different degree of sensitivity of erythrocytes from different rabbits Storage of streptolysin O pneumolysin tetanolysin and θ toxin at 4° C caused a decrease in haemolytic activity However the activity was completely regained after reduction with cysteine

Control Human Sera

The control human sera included some with a normal AST concentration and some with an increased AST concentration which was within the range of titres found after streptococcal infections (Table 1)

An investigation of the neutralization of the other three haemolysins gave the following results (Table 1)

Pneumolysin A low level of antibodies to pneumolysin was generally found whether the AST concentration was high or low These findings indicated that the neutralization of pneumolysin was due to specific antibodies

Tetanolysin Neutralization of tetanolysin was only demonstrated in 3 out of 12 sera with increased AST content and furthermore the A HU content was low Thus these sera did not indicate cross reaction

θ toxin θ toxin neutralizing antibodies were found in nearly all of the 20 sera but the concentration showed a wide variation without any apparent correlation to the AST titre However 3 sera were without any AST content and in these no anti θ toxin could be demonstrated

Human Sera with Unspecific Inhibitors

These sera were titrated with the four haemolysins before and after removal of lipoproteins by dextran (2 4) The unspecific inhibitors neutralized pneumolysin tetanolysin and θ toxin to the same titres as for streptolysin O Their removal resulted in a

immunized horses Ten human sera containing AST active M components were studied for their capacity to neutralize the four bacterial haemolysins mentioned above The results of this analysis are reported in the present paper

MATERIAL AND METHODS

Human serum Serum samples with M-components with AST activity were obtained from ten individuals and were stored at −16 to −20 C The samples contained from 13 000 to 200 000 IU of AST per ml The titres of samples withdrawn from time to time were found nearly unchanged for each of the patients for periods up to 11 years Some characteristics were common for all ten sera 1) The M components belonged to the γG class of immunoglobulins 2) The AST activity was confined to the M components as readily demonstrated by the use of the method which combines haemolysis inhibition and immunoelectrophoresis (14) 3) The apparently monoclonal γG globulins were analogous to immune antibodies as the activity was restricted to their Fab and F(ab') fragments (16) 4) No titre changes were found after precipitation of lipoproteins by dextran (4) which is considered a criterion of specificity (2)

Control human sera Human sera received for routine determination of AST content were used as controls The range of titres were from <25 to 6 000 IU/ml The titres were unchanged or insignificantly changed after dextran precipitation (4)

Human serum samples with a high concentration of unspecific streptolysin O inhibitors (8 11) which were removable by dextran precipitation (2) were used for a few experiments

Three sera from horses were investigated

The antistreptolysin O serum used was Todd's standard serum containing 20 000 IU ml

The anti gas gangrene serum was a commercially available sample (Burroughs Wellcome Gas/Ser batch no 60/81) This preparation contained enzyme treated horse globulins

The tetanus antitoxin was a globulin fraction prepared at this Institute from pepsin treated horse serum

Rabbit red blood cells were only used on the day the blood was withdrawn by heart puncture Blood of individual rabbits was shaken for 10 minutes to prevent clotting Red blood cells were packed by centrifugation and washed 3 times with phosphate buffered saline pH 7 38 Cells were finally packed by centrifugation and a 5 per cent suspension of packed cells was employed This indicator was always used in a volume giving a final concentration of 0 5 per cent red blood cells

Streptolysin O A broth filtrate from a culture of group A streptococci strain S 84 was prepared as described by *Kalbak* (13) and used without further purification In order to ensure complete reduction the streptolysin O was mixed with an equal volume of a neutral 0 067 M solution of cysteine 10 minutes prior to use

Pneumolysin A broth filtrate from a culture of *Dipl pneum* type 6 B was prepared according to the procedure used for the streptolysin O preparation Reduction of the pneumolysin thus obtained was carried out as mentioned for streptolysin O

Tetanolysin The tetanolysin used was a crude tetanus toxin which was obtained by filtration of cultures of *Cl tetani* Albany strain 41 in a semi synthetic medium (5) Toluene was used as a preservative during storage The toluene was removed and the toxin was filtered through a membrane (Schleicher & Schull no 1171) before reduction with cysteine and before the experiments were carried out

θ toxin A commercially available lecithinase C (*Sigma*) was found to contain a sufficient amount of θ toxin for use in our experiments The haemolytic activity of the α toxin (= lecithinase C) was suppressed by the use of a phosphate buffer (14) A stock solution of 10 mg lecithinase C per ml was prepared in phosphate buffered saline containing 50 per cent glycerol A dilution 1 [illegible] (50 μg/ml) was prepared and reduced with cysteine prior to use

CHU One complete haemolytic unit (CHU) of the lysins was defined as the smallest volume which when diluted to 2 ml gave total haemolysis of 0 5 ml 2 5 per cent rabbit erythrocytes The determination was carried out by adding red blood cells to tubes containing two fold dilutions of the lysin The mixtures were incubated for 45 minutes at 37 C and the degree of haemolysis read after intact cells had settled at room temperature The volume containing one CHU of streptolysin O pneumolysin tetanolysin and θ toxin was determined with and without the addition of cysteine in order to follow possible changes during storage

Antistreptolysin O The determination of the number of international units of antistreptolysin O (AST) present in serum samples was carried out with minor modifications of *Kalbak's* method (13)

The *titration* of serum samples against constant doses of the haemolysins was carried out in a similar manner The haemolysins were used in doses of [illegible] CHU As no standard sera were available except for AST the titre of a serum against a particular haemolysin was expressed as the number of complete haemolytic units CHU which were neutralized by 1 ml of serum [illegible]

TABLE 2 *Presence of RSV Neutralizing Antibodies in Five Chickens during Three Successive Periods of Three Months as Determined in the Metabolic Inhibition Test (MIT)*

Chicken no	Virus strain	Period I Antibody titre of		Period II Antibody titre of		Period III Antibody titre of	
		yolk	serum	yolk	serum	yolk	serum
1185	RSV_H	<4*	4	<4	<4	<4	n d
	RSV_{SR}	<4	<4	<4	<4	<4	n d
1201	RSV_H	<4	8	<4	<4	<4	n d
	RSV_{SR}	4	16	4	64	4	n d
1216	RSV_H	<4	n d	<4	n d	32	n d
	RSV_{SR}	4	n d	4	n d	4	n d
1222	RSV_H	<4	<4	<4	<4	<4	n d
	RSV_{SR}	8	8	16	4	4	n d
1234	RSV_H	<4	<4	4	4	<4	n d
	RSV_{SR}	8	64	8	4	4	n d

* = reciprocal titre values
RSV_H = Rous sarcoma virus strain Harris
RSV_{SR} = – – – – Schmidt Ruppin
n d = not done

RESULTS

Table 1 shows the sensitivity of CEF and CAM to our RSV strains of five chickens from flock I during three successive periods of 3 months Each result is based on several experiments As one can see the reactions of CEF and CAM were individually closely related

It is obvious that in this test group two different leukosis viruses interferred with our RSV strains one related to strain RSV_H one to strain RSV_{SR} For instance during the first period the embryos of all chickens but one (1234) were completely resistant to RSV_H However even the embryos of chicken 1234 were apparently infected with a leukosis virus related to RSV_H because they reacted positively in the RIF test During the same period the embryos of all five chickens were sensitive to RSV_{SR} but those of chicken 1216 were positive in the RIF test

There was great individual variability during the three test periods For instance in the first period the embryos of chicken 1201 were completely resistant to strain RSV_H In the second period the embryos were sensitive but the positive RIF test shows that there was still interferring virus present In the third period this chicken produced both resistant and sensitive embryos This variability can hardly be caused by genetically determined resistance since the chickens were litter mates from an inbred line Furthermore as can be seen in Table 1 only exceptionally was there individual variability within one of the periods of the study

During the whole study two chickens 1222 and 1234 produced eggs with antibodies against strain RSV_{SR} as measured in the metabolic inhibition test (Table 2) Similar antibodies were also found in the serum of these two chickens Chicken 1216 continually shed leukosis virus related to strain RSV_{SR} as the positive RIF tests show (Table 1) but no antibody response could be detected This chicken was apparently immunologically tolerant to this subgroup of leukosis virus From Table 2 it can be concluded that it was however immunologically competent In period III it develops antibodies to RSV_H which was shortly after it started producing embryos that showed RIF positivity to RSV_H (Table 1 period II)

reaction by irradiation of the recipient could in some animals lead to a progressive tumour growth if the implanted transformed cells had been grown long enough *in vitro* (5, 20). SV40 transformed mouse cells from continuous lines may also produce tumours in immuno suppressed animals (1, 17).

Vogt & Dulbecco (19) demonstrated an increase in malignancy of polyoma transformed hamster cells during their earlier passages *in vitro*. *Enders* (7) made a similar observation on SV40 transformed hamster cells. The same phenomenon was observed in studies of SV40 transformed rat cells (5). It has also often been shown that a number of different tumours can increase their oncogenic potential by passages *in vivo*.

In the present investigation a metastasizing sarcoma containing SV40 neoantigen has been obtained in adult mouse by making use of the increase in oncogenic potential that can be obtained by prolonged cultivation *in vitro* and *in vivo*. Mouse embryo cells have been transformed by SV40 and cultivated *in vitro* until progressively growing tumours appeared after inoculation of the cells into irradiated animals. A further increase in malignancy giving an easily transplantable tumour was established by passaging the tumour cells into irradiated animals followed by passages into normal ones. In this way cells of the same origin with different oncogenic potential were obtained and studied.

MATERIALS AND METHODS

Virus. SV40 originally obtained from Dr. *D. J. Magrath*, Research Council Laboratories, London, was produced in green monkey kidney cells and titrated in BSC 1 cells. Infectivity of the stock virus employed was $10^{8.5}$ ID_{50} per ml.

Sendai virus with a haemagglutination titre of 1:3 200 was produced in embryonated eggs.

Animals. Inbread CBA mice were used. Male mice 3 to 5 weeks old were chosen except tissue donors which were 2 to 3 months old. The genetic homogeneity was controlled by skin graftings.

Cell cultures. Mouse embryos, kidneys and tumours were aseptically removed, minced and trypsinized. The cells were grown at 3[illegible] C in prescription bottles or Roux flasks with Eagle's medium supplemented with 10 per cent inactivated calf serum and antibiotics.

BSC 1 cells were grown in monolayers in bottles, roller tubes or petri dishes in CO_2 atmosphere employing the same growth medium. They were used for direct tests of infectious virus and for assay of subviral activity by the overlay procedure (9) or by cell fusion with Sendai virus (1[illegible]).

Development of SV40 transformed cell lines. Multiplying cells were infected with SV40 at a multiplicity of about 100. Serial passages were then made once or twice a week from the infected cultures as well as from uninoculated control cultures. The latter did not survive for more than 2 to 5 months. Six to 10 weeks after infection the characteristics of morphological transformation, piling up of cells and nodule formations were observed in the infected cultures. On that occasion nearly 100 per cent of the nuclei contained the SV40 neoantigen demonstrated in immune fluorescence test.

Cell cloning. Dilute cell suspensions were inoculated into petri dishes with small covers[illegible] (14). Those with single cells were marked and later transferred to small petri dishes and left to form lines of single cell origin. The morphology was followed daily under the microscope in a large number of clones.

Tumorigenicity tests and tumour transplantations. After washing in phosphate buffered saline transformed cells were trypsinized, harvested, centrifuged and resuspended in Eagle's medium. The concentration of cells was adjusted with Eagle medium to 50 × 10^{6} cells per ml or some [illegible] other concentrations tested. One ml was injected into the dorsal subcutaneous space of each a[illegible]. On each occasion 3 normal mice and 3 pretreated one day before with 400 r total body x-irradiation were inoculated. They were inspected [illegible] a week and the presence and size of tumours [illegible] recorded. For most animals the observation [illegible] was 3 months. Those with late appearing [illegible] were mostly not moribund within that [illegible] and were therefore followed longer. T[illegible] appeared later than [illegible] months after the [illegible]tion. Animals with tumours were [illegible] and autopsied when they had big tumours and were near to die. At every passage level the t[illegible] from at least one animal was removed [illegible] and divided into 4 p[illegible] one was trypsinized and used for tissue culture, one was fixed in [illegible] cent formalin for histological examination, [illegible] homogenized for assay of CF tumour a[illegible] infectious SV40 and one was minced [illegible] the tumour into another group of animals. [illegible]imately 50 × 10^{6} cells were passed into each [illegible] normal and 3 irradiated mice. When the [illegible] was well established in normal animals the [illegible] treated ones were omitted.

The 50 per cent tumour production dose (TPD_{50}) was estimated at different passage levels by injecting 10 fold dilutions of cell suspensions into groups of indicated number of animals It was calculated according to *Karber* (13)

Storage of cells Tumours were kept frozen at —90 C Cultivated cells were trypsinized suspended in Eagle s medium with 20 per cent calf serum and 10 per cent dimethyl sulphoxide and frozen in liquid nitrogen

Serological tests The animals were bled at autopsy and the sera were kept frozen at —90 C Complement fixation tests were performed with Sever s microtechnique (5) In direct immune fluorescence tests a commercial tagged serum from tumour bearing hamsters was used Indirect tests employed serum from tumour bearing mice and a commercial anti mouse gammaglobulin Before use the mouse sera were pretreated for 1 hour at 37 C with 100 mg of acetone dried mouse liver powder per ml

EXPERIMENTAL AND RESULTS

Tumorigenicity of cell lines carried only in vitro One kidney was removed from altogether 8 mice It was minced grown in tissue culture and transformed by SV40 The transformed cells in amounts of 20 to 50 × 10^6 were inoculated into the respective donors of the kidneys and 2 to 5 mice which all had been pretreated by x irradiation This was done 1 to 6 months after transformation In a few of the animals tested late small regressive tumours appeared but otherwise the results were negative In 2 additional experiments embryos were minced grown and transformed in the same way 50 × 10 cells were inoculated into each of 3 normal and 3 irradiated mice 3 5 6 8 and 9 months after the transformation On the 3 first occasions no tumours appeared later in one cell line regressive tumours up to the size of a walnut were observed among the irradiated animals and after 9 months a progressively growing tumour was obtained in one of the irradiated mice On that occasion 2 of the normal animals showed regressive tumours the size of a rice grain Cells from the other line only gave rise to small regressive tumours in the irradiated animals

The first cell line ME1 and one of the kidney cell lines were chosen for further studies and the other ones were discarded The tumorigenicity of each cell line was tested at 3 to 4 months intervals during two and a half year The ME1 line regularly gave progressively growing tumours in 1 to 3 of the irradiated animals and small regressive tumours in most of the other ones The kidney line was never found to have reached that oncogenic potential Only small regressive tumours in irradiated animals were observed After it had been carried for more than 2 years in vitro additional tests were done twice in 5 irradiated animals receiving as much as 250 × 10^6 cells each Only regressive tumours developed also after these inoculations

Tumorigenicity of cell lines carried in vivo The progressively growing tumour obtained in the ME1 line was passaged in irradiated and normal animals during the first 7 passages and then only in untreated mice In the first 3 passages, progressive growth was only demonstrated in the group of irradiated animals The early passages could therefore be performed only from irradiated mice From the 4th passage progressively growing tumours were obtained in both groups of animals At that passage level the histopathological character of the tumour was also different In the earlier passages it was a well delimited spindle cell sarcoma Later it changed into an infiltratively growing sarcoma with metastases to regional lymph nodes and lungs Metastases were then often but not regularly found and were often seen only by microscopical examination of lymph nodes

Tumour cells from the first 3 passages and every 3rd passage up to the 21st were retested for oncogenicity in normal and irradiated animals after a number of passages in vitro The results were the same as in the original tests cells from the first 2 passages gave tumours only in irradiated mice and the others also in untreated animals The histopathological appearance was also the same as earlier

The results show that the ability to grow in irradiated syngeneic animals may develop during prolonged in vitro cultivation and that it is possible to obtain in vivo and keep

immunizing capacity of some of the cell populations of different oncogenic potentiality.

The importance of the immune response in the present system is difficult to judge. In several animals the tumours developed very late after an early rejection indicating that even small amounts of tumour cells might be able to grow out in an immune reactive host.

At simultaneous injection at different sites of both potentially malignant and malignant cells only the latter grew out. There were no indications that the animals were immunoparalyzed by their malignant cells to a degree that could enable the tumour growth of potentially malignant cells that occurs in irradiated animals.

It is thus difficult to explain the variation in malignancy only as a result of different amount of transplantation antigens or different immunizing capacity. The mechanism behind the increase in malignancy obtained in the present system is still obscure and must be further investigated.

Recently *Kit et al.* (11) described a transplantable sarcoma in mouse which was obtained after inoculation of SV40 transformed mouse kidney cells into adult untreated syngeneic mice. Tumours only developed after prolonged cultivation *in vitro* and transplantations of early tumours were unsuccessful. A tumour excised on the 51st day after inoculation could however be serially transplanted. As in our studies prolonged growth both *in vitro* and *in vivo* seemed to be important for the development of malignancy although passages in irradiated animals were not necessary for an establishment of the tumour. Also their results show that the development of malignancy was not associated with the loss of transplantation antigens.

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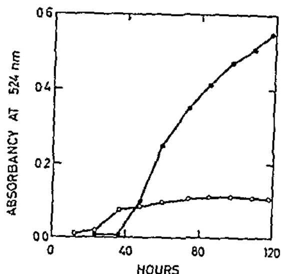


Fig 1 Rate of growth of Ps aeruginosa in medium I with addition of thalidomide (○——○) or choline (●——●) as sole source of carbon and nitrogen

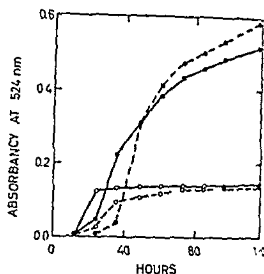


Fig 2 Rate of growth of Ps aeruginosa in medium I containing thalidomide (○ ○) or choline (●——●) Inoculum from medium containing thalidomide (——) or choline)

langen West Germany counting efficiency for ¹ C about 40 per cent) Five ml of ethanol were added to the fractions which were titrated with 0 025 M NaOH using bromcresol green as indicator The phthalic acid peak was rechromatographed on a column of Dowex 2×4 200–400 mesh (chloride form 1 3 × 9 cm) The column was eluted with 0 1 M HCl The fractions containing the radioactive material were pooled and lyophilized using liquid nitrogen for the quantitative removal of HCl

RESULTS AND DISCUSSION

Fig 1 shows the growth curves of *Ps aeruginosa* with choline or thalidomide as the sole source of carbon and nitrogen The inocula were taken from a culture on a choline medium The growth in the thalidomide medium started somewhat earlier than in the choline medium but the growth in the choline medium was found to be much better The growth accelerating effect of thalidomide was studied by the following experiments *Ps aeruginosa* was cultivated in medium I with addition of either thalidomide or choline Inocula were prepared and the numbers of living bacteria in these inocula were determined In the inocula from thalidomide medium the number was 8 × 10⁸ per ml and in the one from choline medium it was 1 5 × 10⁹ per ml Fig 2 shows that the lag phase was shortest when the inoculum taken from the thalidomide medium was transferred into the same medium and longest when an inoculum from choline medium was transferred into the choline medium A growth accelerating effect of thalidomide has been observed previously in *Lactobacillus delbrueckii* (10) The mechanism of this effect is not known

The reason for the poor final growth in the thalidomide medium might be that thalidomide is a poor substrate for growth or that thalidomide is metabolized into a toxic compound The growth was examined in medium I with addition of either choline or both thalidomide and choline The growth obtained was the same in both cases indicating that the poor growth in the thalidomide medium is not due to the formation of a toxic compound from thalidomide

Derivatives of phthalimide are easily hydrolyzed to o carboxybenzoylamino derivatives as the result of a nucleophilic attack by hydroxide ion (6 7) *Schumacher et al* (13) have studied the hydrolysis of thalidomide in buffer solutions at different pH values At 37 °C the hydrolysis was very slow at pH be

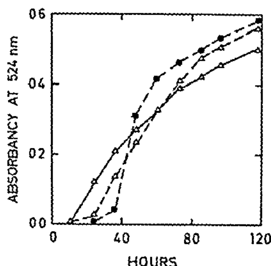
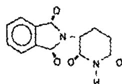
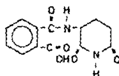


Fig 3 Rate of growth of *Ps aeruginosa* in medium I containing choline (●——●) or both thalidomide and choline (Δ—— Δ) Inoculum from medium with thalidomide (——) or choline (– – –)

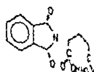
but the rate increased rapidly with incr... pH The first hydrolysis product was ... carboxybenzoylamino) glutarimide (c... pound II in Fig 4) The benzoylamino bo... was fairly resistant to hydrolysis at neutr[al] pH values The possibility of a microbial c... tamination in these experiments was not ex cluded In an enzymic degradation of t... lidomide compounds II III and IV (F... 4) are probable intermediates More than ... per cent of the thalidomide was recov... unchanged after autoclaving a suspen... of thalidomide in water (10) However in a 0... mM solution of labeled thalidomide hydroly... sis was quantitative during autoclaving Th... it is probable that a large part of the di... solved thalidomide is hydrolyzed during au... claving However due to the low solubility of thalidomide the amount hydrolyzed is l... compared to the total amount of thalidomide added To obtain information concerning the possibility that the growth of thalido... might depend on the presence of nonenzym... hydrolysis products *Ps aeruginosa* was ...



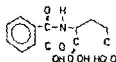
I thalidomide 2 phthalimido glutarimide



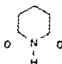
II 2 (o carboxybenzoylamino) glutarimide



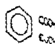
III 2 phthalimidoglutaric a...



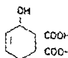
IV 2 (o carboxybenzoylamino) glutaric acid



V glutarimide



VI phthalic acid



VII 3 hydroxyphthalic ...d



VIII 4 hydroxyphthalic acid

F... 4 St...u...t... of compounds related to thalidomide

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Acta path. microbiol. scand. Section B. 78: 495-503, 1970

ISOLATION OF A HERPES-TYPE VIRUS FROM CHICKENS WITH ACUTE MAREK'S DISEASE IN DENMARK

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Isolation *in vivo* and *in vitro* of a strain of Marek's Disease virus (MDV) from chickens with acute Marek's Disease is described. In 6 *in vivo* transmission experiments the per cent of positive chickens varied between 71 per cent and 100 per cent. Between 15 per cent and 75 per cent of the positive chickens showed lymphoid tumours in different organs, chiefly the gonades. In cultures of primary chicken kidney cells foci of refractile cells were produced in four days and microplaques developed in 7 to 9 days. The cell propagated virus proved infectious and oncogenic for day old chickens, and infectious cell suspensions contained cells with herpes like virions in the nuclei.

Marek's Disease (MD) (*Marek* 1907) is an infectious and contagious lympho-proliferative virus disease of young chickens characterized clinically by paresis and paralysis of legs and wings and pathologically by enlargement and infiltration of peripheral nerves and in some chickens also by formation of lymphoid tumours in various visceral organs, especially the gonades, the kidneys, the lungs, the muscles and the skin.

MD was first diagnosed in Denmark by *Andersen* (1935). Since then the disease has occurred with a rising frequency in the autopsy material at the Poultry Research Laboratory, Copenhagen (Fig. 1).

Occurrence of the acute form of the disease (*Biggs et al.* 1965) resembling Chronic Respiratory Disease (CRD) was noticed by *Marthedal* in the early 1960's (*Marthedal* 1965, 1969). The first outbreak was seen as early as 1958 and illegally imported material is suspected of being the origin (*Marthedal* 1969b).

Very recently evidence has been provided that the agent of MD can be propagated in primary chicken kidney cell cultures (*Churchill & Biggs* 1967, *Churchill* 1968), in duck embryo fibroblast cultures (*Solomon et al.* 1968) and in chicken embryo fibroblast cultures (*Vindel* 1964, *Kottaridis et al.* 1968). The agent of MD was furthermore shown to be a herpes virus (*Churchill & Biggs* 1967, *Epstein et al.* 1968, *Nazerian & Burmester* 1968, *Nazerian et al.* 1968) and was found to be strictly cell associated (*Churchill* 1968).

Attempts to isolate a herpes type virus from tissues of chickens with acute Marek's disease in Denmark are reported here.

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Requests for reprints should be addressed to: O. P. Settnes, Department of Tumour Virus Research, Institute of Medical Microbiology, 22 Juliane Mariesvej, 2100 Copenhagen Ø, Denmark.

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TABLE 1 *Results of in vivo Transmission Experiments with Strain CPI L II MDI in Chickens*

In vivo passage number	Inoculum type	Number of chickens Inoculated	Pos./neg.	Per cent chickens positive	Per cent positive chickens with lymphoma	Control Pos./neg. chickens
I	Lymphoma cells	14 (15*)	10/4	71	0	0/15
II A	Whole† blood	13 (15)	13/0	100	46	0/15
II B	Whole blood	24 (25)	20/4	83	15	0/15
II C	Lymphoma§ cells	10 (10)	10/0	100	30	0/10
III	Whole blood	8 (10)	8/0	100	50	0/10
IV	Whole blood	4 (5)	4/0	100	75	0/5
VI	Infected cell cultures	5 (5)	5/0	100	40	0/5

* The bracketed number is the original number of chickens inoculated. The chickens dying within the first 8 days after inoculation are omitted.
† 0.2 ml of frozen whole blood given immediately after thawing without removing the DMSO.
§ Approximately 25 × 10⁶ cells per chickens.
See text for details.

cells. The medium over infected cells has always proved non-infectious for cell cultures and for chickens and it has not been possible to obtain the cytopathic effect in cell cultures of kidneys from any control chicken.

A linear dose response relationship between the number of counted plaques and the number of cells in the inoculum was found, meaning that the single cell is the infectious unit.

Virus in the 6th cell culture passage was tested for infectivity in chickens. 5 chickens, 3 days old, were inoculated with 0.2 ml suspension of infected kidney cells immediately after thawing without removing the DMSO. Chickens were kept as controls. All chickens were observed daily for symptoms. The titre of the inoculum was estimated to be only 2 mPFU/0.2 ml. The results are given in the lowermost part of Table 1.

While the chickens not inoculated remained negative, two of those given infected cells showed respiratory distress after two weeks and by 3 weeks all five showed incoordinated walk and drop of one or both wings. They all appeared very depressed. None of the chickens had died at the end of the experiment, but at post mortem examination all five were positive according to histological criteria and two had furthermore lymphoid tumours in the ovaries. A third was suspected of having a lymphoid tumour in the ovary but the specimen was lost during preparation. Typical foci appeared on monolayers prepared of kidneys from all five inoculated chickens, but none on monolayers of kidneys from the controls.

Electron microscopy. Examination of thin sections of infected cell cultures with the electron microscope revealed particles with hexagonal profiles characteristic of that of herpes viruses in the nuclei (Fig. 7).

The particles were either naked or developed. The naked particles were often without nucleoids or had electron lucent nucleoids and were distributed in the nucleoplasm. In some of the particles an electron dense nucleoid measuring 35-40 nm was seen. The diameter of the naked particles was approximately 100 nm.

Enveloped particles were seen in pockets formed by invagination of the inner nuclear membrane. The diameter of the enveloped particles was 110-150 nm. Few particles have been found also in the cytoplasm.

In a few of the infected cells crystal-like aggregates of smaller hexagonal particles ap-

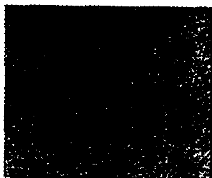


Fig 2 Focus of refractile cells 4 days p i Un stained 100 ×



Fig 3 Monolayer showing foci of refractile cells 5 days p i MGG stained 100 × The blue color is cut away with a blue absorbing filter



Fig 4 Monolayer with a microplaqu 8 days p MGG stained 100



Fig 5 Part of microplaque in figure 4 400 ×



Fig 6 Part of microplaque in figure 4 showing i tranuclear inclusion bodies MGG stained 100 ,

proximately 40 nm in diameter could be seen in the nucleoplasm (Fig 8)

These are believed to be aggregates of core materials Similar aggregates was found by *Stackpole* (1969) in the nuclei of cells infected with the Lucke s frog renal carci... virus Particles of a similar size have al been reported by others (*Epstein et al* 196 *Vaverian* & *Burmester* 1968, *Eidi et al* 1969)

The changes in the cytoplasm gen...

symptoms of MD but not from control chickens kept under isolation conditions. This herpes type virus can be propagated in culture of primary chicken kidney cells and thus propagated virus has proved infectious and oncogenic for day-old chickens. Herpes-like virus particles in the nuclei were seen by electron microscopy of infected cells.

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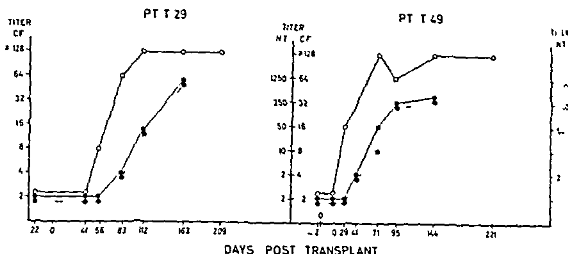


Fig 1 Serological response in two renal allograft recipients with primary CMV infections O——O CF antibodies/Ad 169 ●——● NT antibodies/Ad 169 and ● - - ● NT antibodies/strain T29 and T49 respectively

high CF titres during the remainder of the period of study NT antibodies were not demonstrable in the first serum sample which contained CF antibodies but in the next the NT titre was 2 and from day 83 and thereafter a rising titre was found The serological response in T49 was very similar except that CF antibodies could be registered already 29 days post transplant The first serum sample showing a weak neutralizing effect was from day 41 As can be seen the NT titres measured against the two strains were identical with the exception of a single specimen from patient T49

The course of the antibody response in patient ABN is given in Fig 2 From day 18 [illegible] 260 after the onset of symptoms high relatively rapidly decreasing CF titres were seen There were no NT antibodies in the first serum sample but increasing titre from day 41 to day 95, when it attained a constant level Neither in this patient was there any difference between the titres measured against the two strains

Fig 3 shows the serological response in patients T40 and T27 both of whom had a reactivated infection NT antibodies were present in the pretransplant serum from T40 but not in several sera during the initial phase of immunosuppressive therapy Thereafter the titre rose slowly In several serum samples the NT titre against the homologous strain was approximately twice as high as it was against Ad 169 This patient also had a high CF titre from day 103 to the end of the study period on day 300

The serological response in patient T27 was somewhat at a variance with the response seen in the previously mentioned patients A CF titre of 4 was found in pretransplant serum but the first sample after transplantation was negative From day 76 to day 338

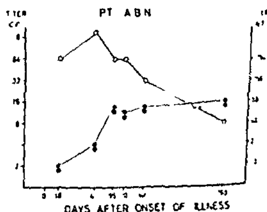


Fig 2 Serological response in an adult with spontaneous primary CMV infection O——O CF antibodies/Ad 169 ●——● NT antibodies Ad 169 and ● ● NT antibodies strain ABN

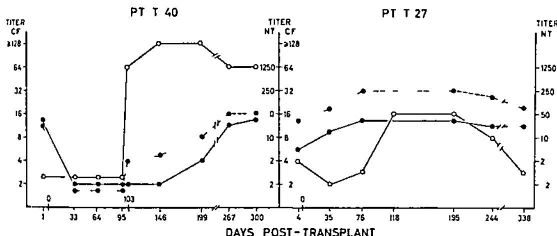


Fig 3 Serological response in two renal allograft recipients with reactivated CMV infections O——O CF antibodies/Ad 169 ●——● NT antibodies/Ad 169 and ●---● NT antibodies/strain T40 and T27 respectively

CF antibodies were demonstrable In general however the titre was low and after day 338 no CF antibodies could be demonstrated All samples contained NT antibodies against both strains and a parallel five fold rise in NT titre took place during the first 80 days of study In all serum samples however re peated measurement showed that the NT titre was about five times greater against the homologous strain than against strain Ad 169

DISCUSSION

CMV infection in these five patients was primarily diagnosed serologically with Ad 169 antigen and later confirmed by the isolation of typical CMV strains from the urine

Using antigens from the different strains the CF titres in each individual serum were found to be of the same order of magnitude One may thus conclude that there is no es sential difference between the CF antigens of the strains tested *Dreesman & Benyesh Melnick* (5) have also shown that there are extensive overlapping between the CF an tigens of various human CMV strains

In the first four patients in this series identical NT titres were demonstrated on parallel titration against the homologous and the heterologous strain The small differences in titre which occasionally were registered are within the limit of error of the method On the other hand, in all sera from the last pa tient the titre against the heterologous strain was five fold lower than against the homo logous strain Since this difference was repro ducible at three separate titrations this patient was obviously infected with a strain of virus which to some degree differs antigenically from strain Ad 169 The two strains must however be relatively closely related as it was possible to demonstrate NT antibodies with antigens prepared from both of them although consistently with about a five fold lower titre against the heterologous strain

Cross neutralization tests with hyperim mune rabbit sera against Ad 169 and the strain isolated from patient T27 confirmed this presumption a cross reaction between the two strains was demonstrated but the two hyperimmune sera both gave a higher titre when tested against the homologous strain (2)

The studies performed here have confirmed previous observations on the temporal dis placement between the rise in CF titre and the rise in NT titre in primary CMV infec tion In cases of reactivated infection the rise in the two types of antibody may occur simultaneously but as in primary in ...

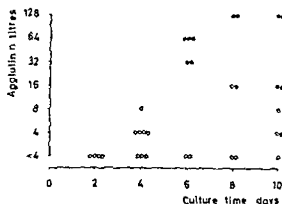


Fig 3 Agglutinin titres in concentrated culture supernatants prepared at harvesting after different culture periods Four culture series containing SRC-stimulated leucocytes (from primed rabbits. Autoradiographic signs of γG immunoglobulin synthesis indicated by closed circles

trated culture supernatants on the 4th day of culturing On the 6th day of culturing agglutinins were present in all culture supernatants except one which had turned alkaline due to a damaged cap On the 8th day of culturing agglutinins were present in all culture tubes containing primed lymphocytes that were tested The maximal agglutinin titre was 256 No agglutinins were detected at any time in the non concentrated culture supernatants of the two series containing non primed leucocytes or in the non stimulated culture tubes containing primed or non primed leucocytes

The concentrated culture supernatants obtained after 18 hr incubation in culture medium containing radioactive amino acids showed anti SRC agglutinating ability as well as autoradiographic evidence of de novo synthesis of immunoglobulins (Figs 3 and 4) It is evident from Fig 3 that the autoradiographic signs of globulin synthesis are less sensitive as signs of antibody production than the liberation of agglutinins into the culture medium However, a positive relation between the two signs of antibody production was found Fourteen out of 15 culture supernatants with agglutinin titres higher than 8 gave autoradiographic signs of immunoglobulin production while only two out of 21 supernatants with agglutinin titres of 8 or less showed immunoglobulin labelling in the autoradiographic preparations Fig 3 also demonstrates that no signs of immunoglobulin labelling were apparent before the 6th day of harvesting while agglutinin liberation was noticed in the concentrated culture supernatants on the 4th day of harvesting

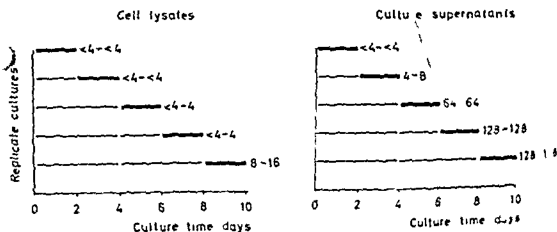


Fig 4 Agglutinin titres in cell lysates and concentrated culture supernatants at harvesting of culture series containing leucocytes from a rabbit previously immunized against SRC

0 2 Days

2 4 Days

4 6 Days

6 8 Days

8 10 Days

Fig 5 Autoradiographs of *immuno* electrophoretic patterns of concentrated culture supernatants from one culture series containing SRC stimulated primed leucocytes and harvested after different culture periods The immuno-electrophoretic patterns corresponded to that shown in Fig 8 Signs of de novo synthesis of γG in cultures harvested after 6 8 and 10 days incubation

The γG precipitation line was the predominating one found to be labelled in the autoradiographic preparations made from the concentrated culture supernatants γG labelling was found in all supernatants which showed any autoradiographic signs of immunoglobulin synthesis In many supernatants γG was the only immunoglobulin found to be labelled with radioactivity (Fig 5) The labelling of γG was most marked in the cathodal (slow) part of the γG precipitation lines In some of the culture supernatants a weak autoradiographic labelling of the γM precipitation line was found in addition to a strong labelling of the γG precipitation line The labelling of γM showed no definite relation to the agglutinin titres In a few culture supernatants after 8–10 days culturing weak autoradiographic labelling of two precipitation lines in the α and β globulin regions was noticed These lines corresponded to the more regularly labelled precipitation lines in the autoradiographic preparations of the cell lysates

Two of the 4 culture series of SRC stimulated primed leucocytes were used for adsorption experiments with measurements of the radioactivity adsorbed out of the culture supernatants with SRC The agglutinins in the culture supernatants were completely removed by the adsorption procedure A positive correlation was obtained between the agglutinin titres in the culture supernatants and the radioactivity bound to the SRC (Fig 6)

The cell lysates prepared after 48 hr incubation in culture medium containing radioactive amino acids contained no or only low titres of anti SRC agglutinins (Fig 7) They showed no autoradiographic signs of de novo synthesis of immunoglobulins However the cell lysates gave autoradiographic evidence of

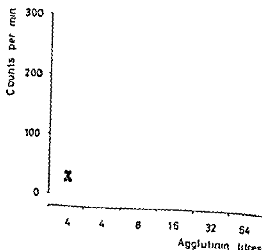


Fig 6 Relation between radioactivity in culture supernatants adsorbed to SRC and anti SRC agglutinin titres in the supernatants

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IMMUNOFLUORESCENCE STUDIES ON KIDNEY BIOPSIES IN ANKYLOSING SPONDYLITIS

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Six patients with ankylosing spondylitis were studied Kidney biop ies were examined by light and fluorescent microscopy Conventional staining revealed vascular changes consisting of deposits in small arteries and arterioles in three of the patients Immunofluorescence revealed pathological changes in all six patients Deposits of immunoglobulin complement and fibrinogen in glomerular and tubular basement membranes as well as in the walls of small arteries and arterioles suggest that immunological processes are associated with ankylosing spondylitis

Extra articular manifestations are characteristic of ankylosing spondylitis (*Hart* 1968) amyloidosis iridocyclitis and aortitis being the commonest of these Recently the frequent occurrence of renal vascular changes in ankylosing spondylitis has been reported (*Pasternack et al* 1970) In the present study immunopathological methods were used for the further characterization of the renal lesion of ankylosing spondylitis

PATIENTS AND METHODS

Six patients with ankylo ing spondyl tis were studied The main clinical data are presented in Table 1 Diagnosis was ba ed on typical featur of the disease early on et painful ankylosis of th spine typical radiological p cture including sacro iliitis and absence of rheumatoid factor Percuta ie ous renal b opsy was performed under local an aesthesia Part of the tissue was fixed in 10 per cent neutral formaldehyde embedded in paraffin was sectioned at 3-5 μ and stained with haematoxylin van Gieson periodic acid Schiff and ilver me thenamine

Part of the renal tissue specimen was snap-frozen and sectioned in a cryostat at 5 μ After drying overnight the sections were wa hed for 10 minutes in phosphate buffered saline (PBS) pH 7 2 Parallel sections were then incubated for 30 minutes with fluorescein labelled a ti human im munoglobulin serum at room temp rature in a humid atmosphere After incubati n the slides were washed in PBS with two changes for 15 min utes and studied when still wet unde a Wild flu orescence microscope (primary filter UG 1 (2 mm) secondary filter GG 13) For the testing of immunofluorescence five fluorescein labelled anti sera were used anti human immunoglobulin se rum (AIg) antihuman complement serum (AC) anti human (fibrinogen serum (AFib) and anti human albumin serum (AAlb) The AIg serum was produced by immunization of one sheep with human IgG and IgM purif ed from serum by immunoadsorption (*L nder* & *Tallbe g* 1970) The AC serum was produced by immunization with zymosan adsorbed C3 according to *Ma diney* & *Muller Eberha d* (1965) The anti fibrinogen serum was produced by immunization of a rabbit with commercially available human fibrinogen (Kabi) The AAlb s rum as produced in sheep by immunization with commercially available hu man albumin (B hringw erke Ag)

The specificity of the antisera used was tested

Received 31 iii 70

TABLE 1 Main Clinical Da[illegible]

Case	Age years	Sex	Duration of disease years	Main clinical feature	Urinary findings
BB	19	♂	½	Spondylitis	–
IK	21	♂	2	Spondylitis Peripheral arthritis	Haematuria
EP	28	♂	6	Spondylitis	
GH	28	♂	9	Tonsillitis Gonorrhea Spondylitis	
PB	38	♂	15	Tonsillitis Spondylitis Peripheral arthritis	–
FM	54	♂	>20	Spondylitis	

by immunodiffusion Conjugation of antisera with fluorescein isothiocyanate was done as described by *Lander & Tallberg* (1960) The fluorescein/protein molar ratio of the conjugates varied from 1.5 to 2.5 Absence of non-specific reactions was confirmed using normal kidney tissue as substrate *The specificity of the reactions was tested by blocking the reactions by prior incubation of sections with unconjugated anti serum*

RESULTS

Conventional staining revealed vascular changes in three of the patients The vascular [illegible]n was localized to the small arteries and arterioles It consisted of segmental or circumferential subintimal deposits sometimes extending through the whole vessel wall The deposits stained strongly purple with periodic acid Schiff and were silver negative The vascular lesions were not surrounded by inflammatory cells An occasional slight increase of interstitial tissue and some atrophic tubules were seen in the three patients who had vascular changes The hilar regions of some glomeruli were strongly PAS positive (Fig 1) No other glomerular changes were noted

The main immunofluorescence findings are summarized in Table 2 Deposition of immunoglobulin and complement was observed in all the biopsies studied

The distributions of immunoglobulin and complement deposition in the glomeruli were uniform from patient to patient The anti serum reacting with IgG and IgM was visible as a smooth linear pattern in the glomerular capillaries The fluorescence was clear but weak in three cases insignificant in two cases and absent in one When present this fluorescent staining affected all the glomeruli in the section studied In contrast to this staining pattern the anti C′ serum reacted wi[illegible] glomeruli to give a diffuse granular fluorescence Characteristically homogeneous [illegible] of fluorescence were observed at the [illegible] (Fig 2) This corresponds to the distr[illegible] of PAS-positive material seen in light micro[illegible]scopy (Fig 1) The fluorescence de[illegible] the presence of complement was always [illegible] and the deposits were well demarcated The fluorescence was granular and similar to that given by the anti C′ serum No stai[illegible] was seen when sections were exposed to a [illegible] albumin serum Patchy staining in the [illegible]

Endogenous creatinine clearance ml/min/1.73 m	Blood pressure mm Hg	Latex	Waaler Rose	Drugs described
89	130/90	–	–	Phenylbutazone
138	100/70	–	–	Phenylbutazone
115	135/95		–	Phenylbutazone
132	125/75		–	Corticosteroids Cyclophosphamide
153	135/90		–	Phenylbutazone
147	130/90	–	–	Indometacine

regions was sometimes seen in glomeruli after exposure to antifibrinogen serum.

Tubular basement membranes stained in three cases. The reaction was very distinct when the sections were stained for C and fibrinogen. Staining for immunoglobulins was weak and in one of the cases where C and fibrinogen were demonstrated no immunoglobulin could be seen. The fluorescence was usually granular and only affected the basement membrane focally (Fig 3). No reaction with the anti albumin serum was observed.

Distinct reactions were seen in kidney vessels when sections were stained for C and fibrinogen. Staining for IgG and IgM was distinct in subendothelial deposits (Fig 4) but was less marked in the walls of small arteries and arterioles where staining for C and fibrinogen was often circumferential and affected the whole vessel wall (Fig 5). In addition to this staining of vessels in three

TABLE 2 *Main Results of Immunofluorescence Tests in Kidney Biopsies from Six Patients with Ankylosing Spondylitis*

Patient	Glomerular basement membrane		Tubular basement membrane		Vessels	
	FAC	FAIg	FAC	FAIg	FAC	FAFib
BB	+	+	+	+	+	+
IK	+	+	+	+	+	
EP	+			—	+	
GH	+	±	–	—	+	+
PB	+	±	–	—	+	±
EM	+	+	+	—	+	+

FAC = fluorescent anti human complement serum
FAIg = fluorescent anti human immunoglobulin serum
FFAb = fluorescent anti human fibrinogen serum


1

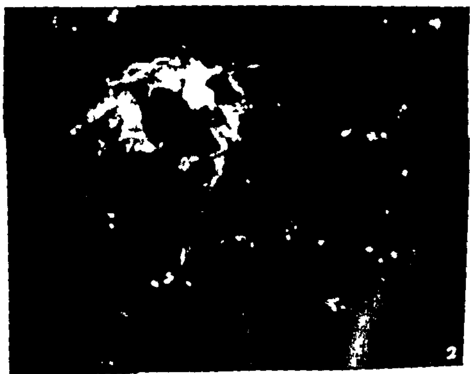

2



Fig 3 Kidney tubules showing deposits of complement (C3) at basement membranes Cryostat section stained with fluorescein conjugated anti human C3 serum × 400

cases there was a distinct staining of intertubular material corresponding to intertubular capillaries (Fig 6) The staining was most marked when sections were stained for C and fibrinogen

DISCUSSION

In a previous article fibrinoid changes in renal vessels in ankylosing spondylitis were described (*Pasternack et al* 1970) The discussion will be focused on the question of whether the results of the present investigation suggest an immunopathological aetiology for these lesions The deposition of immunoglobulins complement and fibrinogen in kidney biopsies has been observed in chronic glomerulonephritis of different origins

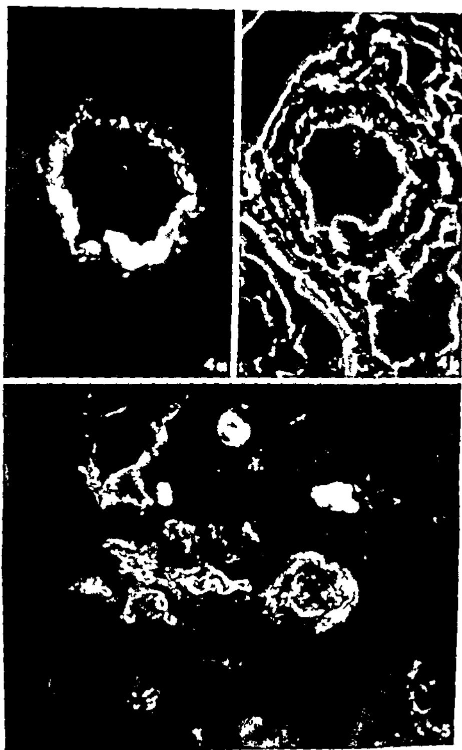
Fig 1 Glomerulus showing deposits of PAS positive material in hilar region Paraffin section × 400

Fig 2 Glomerulus showing lumpy fluorescence in hilar region Cryostat section stained with fluorescein conjugated antihuman C3 serum × 400

(*Mellors et al* 1957 *Koffler & Paronetto* 1965 *Hadley & Rosenau* 1967 *Koffler et al* 1967 *Lerner et al* 1967 *Dixon* 1968) In the cases of ankylosing spondylitis studied no clinical or light microscopic signs of glomerulonephritis were observed This agrees with previous findings in ankylosing spondylitis (*Hollander* 1966)

The immunohistological studies on kidney biopsies from our six patients demonstrated the glomerular changes usually associated with glomerulonephritis deposition of immunoglobulin complement and fibrinogen However the glomerular deposits were slight and contrasted with the marked changes observed in the small arteries and glomerular arterioles The slight glomerular deposits observed by immunofluorescence were not associated with the changes revealed by conventional staining It seems probable that they reflect the general process affecting small arteries

The discrepancy between the strong staining for complement in a granular fashion


4a
4b
5

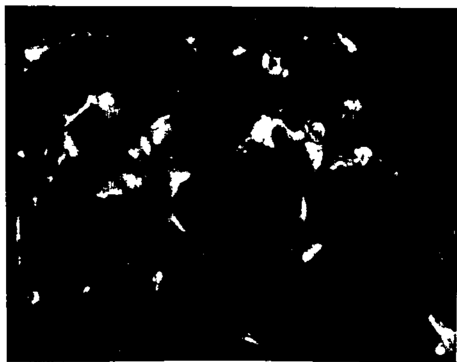


Fig 6 Staining of intertubular material corresponding to intertubular capillaries Cryo stat section stained with fluorescein conjugated antiserum against human C 3 × 400

and the weak linear staining for IgG and IgM deserves some comment as an immune reaction involving antibody and antigen ought to bind complement at the same site As discussed by *Hadley & Rosenau* (1967) the initial treatment of the cryostat sections is of great importance to exclude the possi bility of false po itive reactions due to the retention of soluble unreacted serum protein in the sections The procedure used by us involved washing the cryostat sections for ten minutes in phosphate buffered saline

Fig 4a Staining of subintimal deposits in an inter lobular artery stained w th fluorescein conjugated antiserum against human IgG and IgM Cryostat section × 400

Fig 4b Same vessel as in F g 4a as seen in phase contrast microscopy Note the relation of deposit to internal elasti membrane (arrow) × 400

Fig 5 Staining of deposits extending through the whole vessel wall Subintimal deposits are seen to occlude three mall vessels Fluorescein conjugat d anti human C 3 erum × 400

prior to incubation with fluorescein labelled antiserum and the adequacy of this treat ment was verified by tests with normal kidney tissue obtained at surgery in which no staining was seen with any of the anti sera used It is therefore assumed that the linear fluorescence observed when sections were stained for IgG and IgM is due to ac tive deposition of immunoglobulins at the glomerular basement membranes

The different distribution of complement may be explained by the observation by *Feldman et al* (1966) that the distribution of IgG and complement in glomeruli seen in kidney biopsies from children suffering from post streptococcal glomerulonephritis was dependent on the stage of the disease. During the acute phase granular distribution of both IgG and complement was anatomically similar involving the capillary basement membranes and the mesangial zones During the healing phase on the other hand depo sition of fluorescent granules for IgG along capillary walls was replaced by a weak linear

staining while *complement* was found predominantly in a mesangial pattern. The duration of disease in our patients varied from 6 months to more than 20 years and there was no positive or negative correlation between duration of symptoms and intensity of *immunofluorescent staining*. However, the biopsies from the two patients exhibiting peripheral arthritis also showed the most distinct glomerular and tubular BM fluorescence when stained for IgG and IgM.

Linear immunoglobulin deposits at the glomerular basement membrane (GBM) have been attributed to the presence of antibodies in the circulation reacting with an autoantigenic BM antigen (*Feldman et al* 1966, *Lerner et al* 1967, *Dixon* 1968, *McPhaul & Dixon* 1969). The possible relationship between the autoantigenic GBM antigen(s) and connective tissue antigens at other sites is suggested by the demonstration of the widespread distribution of the antigen responsible for the development of experimental Masugi nephritis (*Va asaka & Shibata* 1969). In ankylosing spondylitis exposure of potentially autoantigenic determinants cross reacting with GBM antigen(s) might occur following vascular and joint injury. In contrast to the relatively slight glomerular changes observed vascular lesions seem to be a frequent finding also demonstrable by light microscopy (*Pasternack et al* 1970, *Ball & Hathaway* 1966, *Smythe* 1966). Although in our cases and in the cases previously studied by *Pasternack et al* (1970) the vascular changes in the kidney vessels were unaccompanied by any cellular infiltrates, focal deposits of lymphocytes and plasma cells have been described in association with the cardiac lesions, aortitis and vascular changes adjacent to affected joints in ankylosing spondylitis (*Graham & Smythe* 1958, *Engfeldt et al* 1954). These observations in association with the present immunohistochemical findings of complement, fibrinogen and occasionally immunoglobulin at the site of the vascular lesions emphasize the difficulty of differentiating between the vascular changes in ankylosing spondylitis and the vasculitis seen in other diseases, notably autoimmune diseases (*Paronetto* 1968). Although the immunohistochemical changes observed do not prove that the formation or deposition of immune complexes is responsible for the vascular lesions, the results indicate that immunological phenomena are associated with them. The immunohistochemical findings in vessels and glomeruli suggest that immunological processes are involved in the natural history and possible in the aetiology of ankylosing spondylitis.

We thank *Dr I J Linen* of the Institute of Serology and Bacteriology for providing the anti fibrinogen and the anti IgA sera respectively. Dr *J Marto* of the Rheumatism Foundation Hospital Heinola kindly remitted five of the patients for which we thank him.

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BRIEF REPORT

CORRELATION BETWEEN THE FIMBRIATED STATE AND COMPETENCE OF GENETIC TRANSFORMATION IN *MORAXELLA NONLIQUEFACIENS* STRAINS

Kjell Bøvre and *Leif Oddvar Frøholm*

It was recently reported that colony variants of *Moraxella nonliquefaciens* (2) are characterized by distinct electron microscopical features (1) One colony form is spreading corrodes the agar and consists of cells which often possess fimbriae (the SC type) whereas the normal appearing form contains only a few and distinctly fragmented fimbriae (the N colony type) Among SC descendants an intermediate weakly corroding and generally non spreading but *fimbriated* variant has been observed (NSC) which is unstable in the sense that about one per cent of the colonies contain revertants to the typical stable SC form. In addition one can find variants in SC cultures which apparently have lost the spreading and corroding character except for very infrequent reversal to SC The latter forms are called N_p in the present communication i e not permanent N They are very similar to the permanent N form N_{np} re-

Received 29 xi 70 *from Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo Rikshospitalet Oslo Norway and Statens institutt for folkehelse Oslo Norway*

TABLE 1 *Comparison of Competence in Colonial and Fimbriation Variants of* Moraxella nonliquefaciens *Strains*

Colonial and fimbriation variants	Strains transformant yield in per cent of the transformant number elicited in autologous SC recipient				
	3067/66	3832/66	3179/66	7784	8 6 1
N_p	0b				0b
N_{np}	<10[illegible]	0 02	0 [illegible]	0 1	
N_p + SC				30d	
NSC	100				
SC	100	100	100	100	
	(10 [illegible] 10 [illegible])	(10 [illegible] 10 [illegible])	(10 [illegible] 10 [illegible])	(5×10	

N_{np} Non spreading non-corroding colonies no fimbriae observed by electron m[illegible]
N_p Spreading-corroding colonies very infrequently observed fimbriae detected [illegible] after selection of the SC form
NSC Generally weakly corroding colonies fimbriae detected without selective measures High frequency of variation to the SC form
SC Typical spreading-corroding colony form with easily observed fimbriae in all electron microscopical preparations.
a Quantitative streptomycin resistance transformation (1) with identical M non[illegible] DNA in the compared parallels
b No transformants detected by sensitive long term (continuous) DNA exposure technique [illegible]
c Higher proportion (10 100 per cent of spreading-corroding colonies among [illegible] by 3067/66 N_p DNA than among recipient colonies < 0 003 0 1 per cent)
d The mixed recipient arose in each non selective subcultivation of strain [illegible] different preformed variant colonies Most transformants were [illegible]
e Proportion of transformants to recipient cell numbers in short term [illegible] DNA [illegible]

TABLE 2 *Relationship between Competence and Colony Type in Non Selected Recipient Cultures of* Moraxella nonliquefaciens

Strains conditions (A and B)	Per cent SC colonies in the recipient population[b]		Number of transformants	Per cent SC colonies in the transformant population[b]	
	Mainly corroding	Clearly spreading		Mainly corroding	Clearly spreading
3835/66 A	2	1	20 000	0	30
3835/66 B	0	0	0		
3438/66 A	40	0 1	15 000	95	5
3438/66 B	40	0 1	10 000	70	10
3484/66 A	100	0 2	10 000	80	10
3484/66 B	1	0	1 000	0	95
3576/66 A	100	0	[illegible]0 000	90	10
3576/66 B	1	0	400	0	100
3085/66 A	0	0	0		
3085/66 B	0	0	0		

A Culture from recently opened ampoule of lyophilized strain

B Corresponding culture (see A) after months of non selective subcultivation on blood agar plates kept at room temperature for 7–14 days between inoculations

Long term (continuous) exposure to DNA extracted from a streptomycin resistant mutant of 3067/66 N_p (permanently non spreading and non-corroding) approximate numbers of resistant transformants obtained with comparable numbers of exposed cells and examined plates The transformation frequency of the first four A cultures in simultaneous quantitative transformations (20 min DNA exposure) was in the order of 10 –10 in relation to exposed cells For transformation techniques see previous references (1)

[b] Four days of incubation of blood agar cultures in a humid atmosphere at 33 C approximate figures The corroding character was recognized by studying the agar after scraping off the colonies The corrosion as well as the spreading often comprised only part of the colony which could complicate the assay

presented by the extensively studied 3067/66 N substrain (1) It is virtually impossible to detect SC type fimbriae in N_p without the use of selective measures as shown by electron microscopy of the substrains 3179/66 and 3832/66 N_p previously designated N although they showed evidence of not being permanent (*op cit* and *vide infra*)

The results of the present study show that with in strains of *M nonliquefaciens* the occurrence of the fimbriated SC and NSC forms is positively correlated with competence in genetic transformation This novel finding is preliminarily reported

Eleven strains or sub trains of *M nonlique faciens* were studied 3067/66 N_p N_p NSC and SC 3179/66 N_p and SC 3832/66 N_p and SC 7784* N_p and SC and 826/61 N_p In addition several isolates of this species were subjected to less comprehensive examination All the organisms grew as soft easily emulsifiable colonies The

* Strain number in the National Collection of Type Cultures London

transformation and electron microscopical techniques were the same as before (1)

The results partly presented in Tables 1 and 2 can be summarized as follows (a) There is a high correlation between the fimbriated SC colony state and the competence in genetic transformation Both the relative proportion of such colonies and the general transformability of a strain tend to decrease by serial non selective subcultures on blood agar Recipients of the non fimbriated N_p type were non transformable in sensitive tests It is relevant to note that 3179/66 N_p after prolonged growth in broth formed a barely visible rim on the glass tube just at *the surface of the fluid* In subculture on blood agar this film revealed fimbriated SC cells which had the same high degree of competence as the corresponding original SC substrain (b) There is a *clear increase in the* proportion of SC colonies (particularly of those with pronounced spreading) among streptomycin resistant transformants elicited by DNA from N_p cells suggesting a selection of the fimbriated SC cells among the recipient N_p *cell populations* Some of the trans

formant colonies appeared normal when assayed but to the extent they were checked proved to be corroding or spreading after prolonged incubation or subculture

The correlation between fimbriation and competence indicates a role of the fimbriae in transformation of these strains However the different degrees of transformability in the respective SC cell populations (Table 1 bottom line) have to be explained An exact estimation of the proportion of fimbriated cells in a culture would require extensive electron microscopical studies The 7/84 and 3179/66 SC populations may contain a larger proportion of fimbriated cells than do the 3067/66 and 283./66 SC cultures On the other hand a difference in fimbriation corresponding to a 100–1000 fold difference in competence would probably have been discovered during the electron microscopical investigations performed (ref 1 and previously unpublished experiments) unless some special fimbriae are concerned with transformation and their presence not parallelled completely by the total number of fimbriae observed The fimbriae located on restricted areas of the bacterial surface could thus be important It would seem likely however that competence in *M nonliquefaciens* is a complex system depending on several factors as appears to be the case in other organisms (3) The fimbriae may even take no part in the transformation process but merely be associated with competence as another reflection of the cellular physiology in the competent state It is not known whether fimbriation could develop or increase as a *consequence* of DNA uptake or *transformation* in *M nonliquefaciens*

Further studies are needed to decide among the various possibilities indicated above The association between fimbriation colony form and competence appears to be a rational starting point for studies on the nature of competence in these strains. The discovery has already found practical use in transformation work since it permits maintenance of transformability at a high level in strains which tend to lose competence

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THE HL-A SYSTEM
GENETIC AND BIOLOGICAL IMPLICATIONS

JEAN DAUSSET

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This paper includes lectures given in February 1970 at Rikshospitalet Oslo The Karolinska Institutet Stockholm The Institute for Medical Microbiology University of Lund and at The Danish Society of Pathology Copenhagen

The subject of this presentation will be the genetics of the main histocompatibility system in man *i e* the HL A system I shall thus consider the theoretical and practical aspects of the choice of a suitable donor and also try to discuss the physiological role of this system which is maintained in its diversity not in order to irritate the surgeons but for another and probably more important purpose.

The HL A system appears to be the most complex genetic system ever known in man It is the equivalent of the H 2 system in mouse and of other main histocompatibility systems known to exist in all other species studied

All the work made in man was possible because of the precise and patient research made in animals and particularly in mice by *Gorer Snell* (46) The link between mice and men was effected by *Amos* (1) when by means of leuco agglutination he discovered that the H 2 antigens are also present in leucocytes

The HL A system has been progressively defined by the joint efforts of several teams all over the world After our demonstration in 1958 of the first leucocyte antigen Mac (16) *Van Rood* found the contrasting distribution of two other antigenic entities 4a and 4b (52), and *Payne & Bodmer* (39) described an allelic pair of antigens LA1 and LA2 (the latter being in fact the antigen Mac) Since then the contributions of other teams namely *Shulman Amos Walford Terasaki Ceppellini Lalezari Batchelor* and in Scandinavia *Kissmeyer Nielsen Thorsby and others* have amassed a large amount of data and have accelerated the definition of tissue antigens The role played by the workshops, so well organized by *Amos Van Rood Ceppellini & Terasaki* must be emphasized (41 2 13 49)

In the history of human blood groups the role played by the associations found between antigens during population studies was extremely important For example the antigens C D and E of the Rhesus system are at least in the Caucasian population frequently associated Antigens M and S in the MNS system are also frequently associated

The same kind of work has been carried out for leucocyte antigens and on the basis of the correlations observed in population studies, with *Ivanyi* in 1965 we came to the conclusion that all these antigens belonged to a single system that we proposed to call Hu 1 (17) Now this system is internationally known as the HL A system

The present situation of the HL A system slightly modified after the Fourth Conference on Histocompatibility held in Los Angeles

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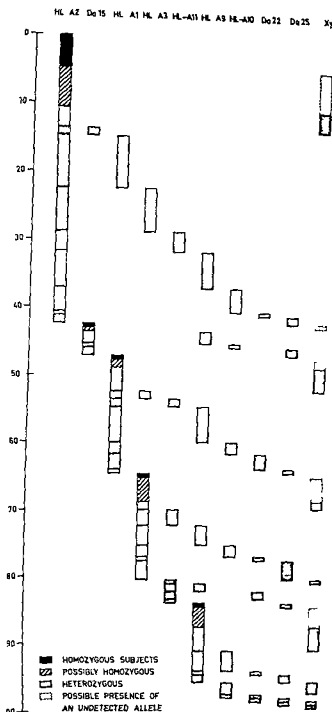


Fig 2

Da 15 and the gene of the second locus present on the other chromosome HL A12

It is not yet possible to establish with certainty the rate of recombinations between the two loci But it seems to be relatively high perhaps about 1 per cent On the whole already six families with crossing over have been observed in the world (54) 186 families were studied in our Laboratory We could determine 20 HL A antigens for 85 of them and 26 HL A antigens for 67 others

With the newly discovered antigens only 2

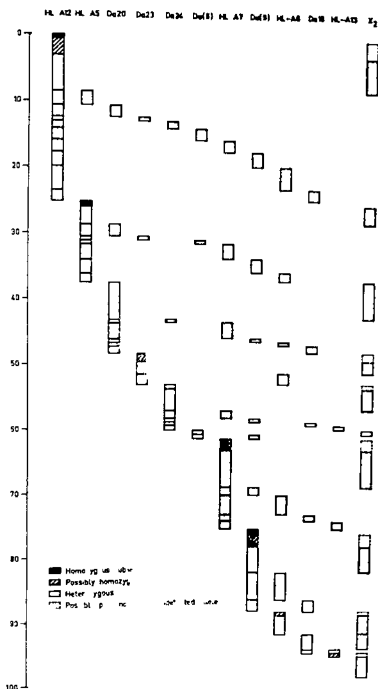


Fig 3

per cent of the genes of the first locus and 6 per cent of the genes of the second locus are still unknown at least in our hands (Table 1)

Table 2 shows the repartition of the 340 observed haplotypes in the 85 families studied for 20 HL-A antigens This number is very near the expected one It is to be noted that there are some haplotypes which are clearly more frequent than the others. For instance *HL-A1 8* was observed 20 time in 170 in

TABLE 1 *Segregation of HL Specificities in + − × − − Matings*

	Children positive	Children negative
HL A 1	93	113
HL-A 2	154	147
HL A 3	42	35
HL-A 9	102	103
Da 15	64	51
Da 17	64	39
Da 21	22	26
Da 22	23	17
Da 25	17	23
Da 4	115	121
HL A 5	75	66
Da 20	31	35
Da 23	23	11
Da 24	12	10
HL A 7	97	102
Da (9)	26	26
HL A 8	17	10
HN	5	3

sibility of the existence of a gene analogous to the T locus in mice closely linked to H2 which would induce sterility

Finally we have demonstrated that the HL A system was independent of many other biological markers erythrocyte platelet serum systems and erythrocyte enzyme system We must add that no close linkage with any known normal or pathological gene has been found as yet

Thus in spite of the extreme polymorphism of this genetic system it is possible to reach a logical arrangement which perfectly follows the mendelian laws It has taken a long time and it has necessitated hard work to demonstrate this genetical logic The difficulty was mainly due to a very peculiar feature of the HL A gene products

The HL A region is probably composed of at least two closely linked loci with a 1 per cent recombination fraction Each locus is a functional unit with several possibilities of mutations The direct or indirect product of this functional unit would be a *molecule* with several antigenic determinants or factors The allelic products of the same locus would differ by one or several modifications of these factors

The serological complexity of the HL A system is probably due to at least two phenomena 1) antigenic factors (still hypothetical), 2) cross reactions between the different products of a same locus (well established fact)

In a given population, it seems that some antigenic factors are so frequently associated that it is impossible to dissociate them when the absorption is carried out with cells from the same population However one may expect that other populations include individuals who lack one or several of these factors The dissociation would then be possible (17)

Thanks to the discovery, independently made by *Colombani* (18, 15 14) in our Laboratory and *Svejgaard* (15) in Denmark it is known that the greatest part of the serological complexity is due to the extreme frequency of cross-reactions between the antigenic products of the same locus

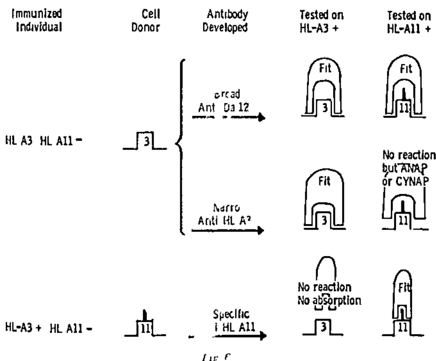
No cross reaction between products of the two loci has been observed as yet This seems to indicate that they have different ancestor genes

It must be assumed that the common part between allelic products is large enough to allow cross reactions between them Cross reactions explain the development of broad antibodies reacting with several allelic products thus included in broad specificities

They explain also the development of what we propose to call narrow antibodies which react with one specificity but are absorbed without reaction by another leading to the ANAP phenomenon (Agg negative absorption positive) described by *Van Rood* (59) (Fig 6)

The first example observed of cross reaction was that between HL A2 and Da 15 or Ba* (18) Later on the anti Da 2 broad antibody was shown to react with both HL-A2 and Da 15 which are two allelic of the first segregant series

Cross-reactions between products of the second series are even more striking In 1965 an antigen Da 6 was described but last year we discovered that Da 6 could be broken into



3 specificities all being alleles of the 2nd locus HL A5 Da 19 (LND) and Da 20 (R*) (14) This year Da 19 was itself broken into two other specificities Da 23 and Da 24 which are completely included in Da 19 (15) (Fig 7)

These cross-reactions are of the utmost practical importance for otherwise it would probably be impossible to find a compatible donor the number of combination being much too large

We shall come back to this point which is extremely important from the practical angle

The chemical analysis of the HL A antigens are now in progress in several Laboratories (28 43 37) We are working on this problem in collaboration with *Allen Davies* (28)

The striking analogy of H 2 and HL A soluble antigens has already been underlined by several authors It has been shown that HL-A soluble antigens are glycoproteins with 92 per cent of proteins and 8 per cent of neutral sugar Their molecular weight is 50 to 60 000 According to *Mann s & Nathenson s* work (37) it seems that the two loci build different molecules which could be separated by ion chromatography and acrylamid gel electrophoresis This is an argument in favour of the concept of two functional units on the chromosomes

More clear cut chemical data are needed before we can express any definite opinion about the relationships between genes and final products However we may at least give our present concept of the HL A system

It is quite possible that the parts of the locus governing the main determinants which behave as alleles in the Caucasian population are pseudo alleles in fact and it would not be surprising at all if two of these main determinants were found to be governed in CIS position by the same chromosome in another population

Now I should like to tell a few words about the localization of these substances They are

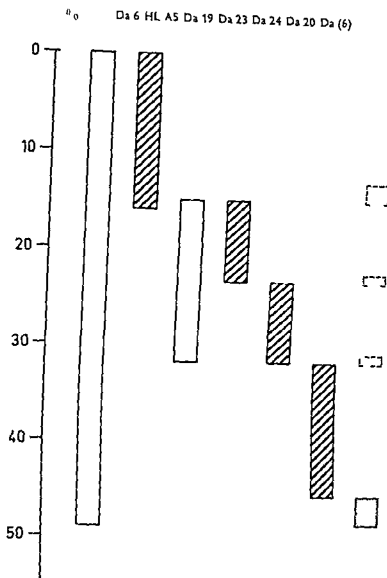


Fig 7

components of the cellular membrane of most of the tissues It has been possible to visualize their presence

Anti HL A antigens have been labelled with ferritin by *Kourilsky & Levy* (14) The electron microscopic section of lymphocytes shows that as for the H 2 model H L A antigens are gathered in distinct areas They are not uniformly distributed on the surface The biological significance of this fact is still unknown

The localization on the different tissues of the body has also been studied but this time by absorption experiments Antigen HL A2 (Mac) is present in huge quantity in spleen and in decreasing quantities in liver lungs kidney heart intestine and aorta However from these preliminary data it is not possible

TABLE 4

MIXTURE OF ANTIBODIES AGAINST

ANTIGENS IN CIS, ALLELIC OR TRANS POSITION

Genotype of the donor's spermatozoa	HL-A 1 8 / HL-A 2 5	
		% of cytotoxicity
Each antibody alone		48 to 59
Mixture in CIS	HL-A 1 + 8	35
	HL-A 2 + 5	42
Mixture in allelic or TRANS	HL-A 1 + 2	73
	HL-A 5 + 8	68
	HL-A 1 + 5	67
	HL-A 2 + 8	78

to conclude that heart for example is more easily transplantable than liver or spleen (7).

The localization of HL-A antigens on spermatoza was studied by cytotoxicity technique. The haploid expression of HL-A genes on spermatozoon was probably demonstrated.

Table 4 gives an example of this study. The genotype of the donor's spermatozoa was HL-A1 8/HL-A2,5. Each of the antibodies against these specificities when used individually gave a percentage of cytotoxicity of around 50 per cent. When we mixed the antibodies against the product of the two genes in CIS position (anti HL-A1 + anti HL-A8 or anti HL-A2 + anti HL-A5) the percentage of killed spermatozoa remained around 50 per cent. On the contrary, when the mixture of antibodies was against product in trans or allelic

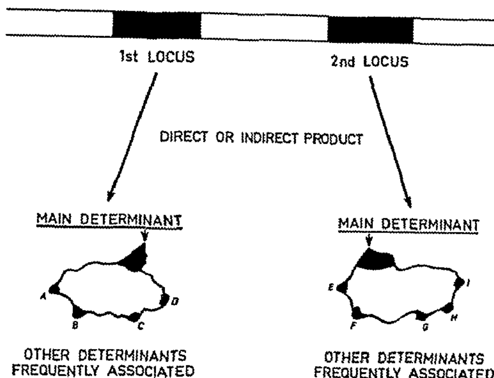


Fig 8

position for example anti HL A1 + anti HL A2 the percentage of killed spermatozoa rose up to 72 to 81 per cent The same experiment was made with another donor Here again the mixture of antibodies against products of genes in CIS position gave less than 50 per cent of killed spermatozoa The mixture against antigens in trans or allelic position gave from 67 to 78 per cent of killed spermatozoa A specific anti HL A2 against the spermatozoa of individuals who were homozygous for HL A2 gave a percentage of killed spermatozoa around 80 per cent (29)

The finding of a probable haploid expression in spermatozoa makes available the first mammalian material of this kind We have also found the same haploid expression of H 2 in mice spermatozoa If this haploid expression is confirmed the direct determination of the haplotypes and genotypes of the HL A system in males will be possible It will also be possible to study directly the *rate* of recombination and the *rate* of mutations One can also contemplate the possibility of gametic selection based on the use of cytotoxic antibodies recognizing HL A or other antigens One might even speculate on the prevention of hereditary diseases governed by linked loci through artifical insemination with one population of spermatozoa

The other biological implications of the knowledge of the HL A system are numerous.

It is certainly one of the best markers available because of its extreme polymorphism. Preliminary data indicate that it will be very useful in anthropology For example oriental populations are practically devoid of antigen HL A1 Other antigens, for instance Da 15 or Ba* is much more frequent in Negroes than in Caucasians It is probable that some antigens exclusive to some populations will be found

It seems to be of utmost importance to establish the world HL A map before the disappearance of isolated population This will be the subject of the next Workshop on Histocompatibility

But attention is now focused on two points which are the role of HL A in transplantation and the role of HL A in the defence of body integrity and in the susceptibility diseases

Regarding transplantation almost every thing has been said Though the role of HL-A in transplantation is widely accepted a certain degree of scepticism remains mostly among surgeons but apparently not in Scandinavia the scepticism being mainly due to the difficulty to correlate the fate of grafts between unrelated individuals with typing Obviously the best results were observed with grafts between related individuals For example in skin grafts performed between sibs by *Amos* (3), or by *Ceppellini* (11), the longest survivals (21 days) were observed when the two sibs had received the two same HL A haplotypes from their parents When they had one haplotype only in common the survival was 14 days and when they differed for the two haplotypes the survival was only 13 1 days

This observation was confirmed by results of mixed lymphocyte cultures *Bach* & *Amos* (5) could predict the existence of a single main system in man when they observed that there was no stimulation between the lymphocytes of 25 per cent of the sibs studied that is to say those who were HL A identical

We made the analysis of 156 kidney transplantations performed in Paris Obviously the best ones are those performed between HL A identical sibs. All the 24 cases are still in perfect condition (27 31) These data are converging proofs that the HL A system is the main histocompatibility system in man that the other histocompatibility systems are weak having no activity in MLC test or under immuno-depression However there are other systems undoubtedly, since skin grafts are nevertheless rejected between HL A identical sibs

The situation is not so good in HL-A haplo identical situation, that is to say when one haplotype is shared by donor and recipient (grafts performed between HL-A haplo-identical sibs or between child and parent) Here the possible presence of incompatibilities governed by the haplotype which is not shared is sufficient to shorten the survival time of either skin or kidney grafts In our statistics 86 per cent of success are counted

The results are less good in HL-A different situation between unrelated individuals when donors and recipients differ on the two HL-A chromosomes Here only 56 per cent of survivals are observed. The fact that the results are directly related to the number of HL A haplotypes genetically common to donors and recipients is striking (27 37)

A direct correlation between the number of incompatibilities and the fate of kidneys has also been found The maximum of HL A incompatibilities between two individuals is four (2 loci on two chromosomes) 85 per cent of the grafts with 3 incompatibilities out of 4 were rejected 55 per cent with two incompatibilities 22 per cent with one incompatibility, and 6 per cent with no detectable incompatibility

It is now urgent to determine whether some HL A antigens are more immunological than others For this purpose *Felix Rapaport* and I have carried out an experiment of skin grafts performed from children to fathers (25 26) The father was grafted with two skins coming from two children each of them having received one of the two different maternal haplotypes C or D 238 grafts were made We observed that the skins were always rejected in a bimodal fashion For example the skins bearing the C haplotype were rejected in 10 days and those bearing the D haplotype in 13 to 14 days This also indicates that the HL A system is the main transplantation system for even when they are identical for the HL A system sibs may receive different sets for the other H systems.

The mean survival time of the skin allografts for the HL A compatible grafts was 15 10 ± days in contrast with 12 1 ± days for the incompatible grafts ($p < 0\ 01$)



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to conclude that heart for example is more easily transplantable than liver or spleen (7)

The localization of HL A antigens on per... ma...oza was studied by cytotoxicity technique. The haploid expression of HL A genes on spermatozoon was probably demonstrated.

Table 4 gives an example of this study. The genotype of the donor's spermatozoa was HL A1,8/HL A2 5. Each of the antibodies against these specificities when used individ... gave a percentage of cytotoxicity of ... 50 per cent. When we mixed the anti... against the product of the two genes ... position (anti HL A1 + anti HL A8 ... HL A2 + anti HL A5) the percentage of ... ed spermatozoa remained around 50 ... On the contrary, when the mixture ... bodies was against product in trans ...

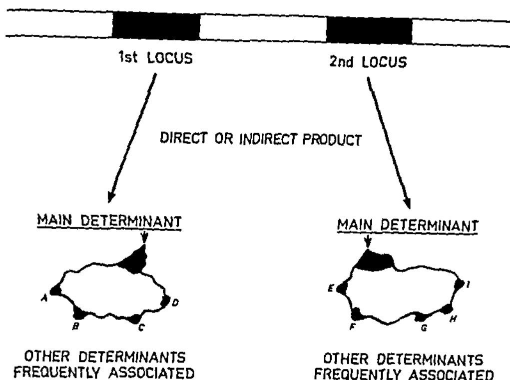


Fig 8

position for example anti HL A1 + anti HL-A2 the percentage of killed spermatozoa rose up to 72 to 81 per cent The same experiment was made with another donor Here again the mixture of antibodies against products of genes in CIS position gave less than 50 per cent of killed spermatozoa The mixture against antigens in trans or allelic position gave from 67 to 78 per cent of killed spermatozoa A specific anti HL A2 against the spermatozoa of individuals who were homozygous for HL A2, gave a percentage of killed spermatozoa around 80 per cent (29)

The finding of a probable haploid expression in spermatozoa makes available the first mammalian material of this kind We have also found the same haploid expression of H 2 in mice spermatozoa If this haploid expression is confirmed the direct determination of the haplotypes and genotypes of the HL A system in males will be possible It will also be possible to study directly the *rate* of recombination and the *rate* of mutations One can also contemplate the possibility of gametic selection based on the use of cytotoxic antibodies recognizing HL A or other antigens One might even speculate on the prevention of hereditary diseases governed by linked loci through artifical insemination with one population of spermatozoa

The other biological implications of the knowledge of the HL A system are numerous.

It is certainly one of the best markers available because of its extreme polymorphism Preliminary data indicate that it will be very useful in anthropology For example oriental populations are practically devoid of antigen HL A1 Other antigens for instance Da 15 or Ba* is much more frequent in Negroes than in Caucasians It is probable that some antigens exclusive to some populations will be found

It seems to be of utmost importance to establish the world HL A map before the disappearance of isolated populations This will be the subject of the next Workshop on Histocompatibility

But attention is now focused on two points which are the role of HL A in transplanta tion and the role of HL A in the defence of body integrity and in the susceptibility dis eases

Regarding transplantation almost every thing has been said Though the role of HL A in transplantation is widely accepted a certain degree of scepticism remains mostly among surgeons, but apparently not in Scandinavia the scepticism being mainly due to the diffi culty to correlate the fate of grafts between unrelated individuals with typing Obviously the best results were observed with grafts be tween related individuals For example in skin grafts performed between sibs by *Amos* (3) or by *Ceppellini* (11) the longest surv ivals (21 days) were observed when the two sibs had received the two same HL A haplo types from their parents When they had one haplotype only in common the survival was 14 days, and when they differed for the two haplotypes the survival was only 13 1 days

This observation was confirmed by results of mixed lymphocyte cultures *Bach & Amos* (5) could predict the existence of a single main system in man when they observed that there was no stimulation between the lym phocytes of 25 per cent of the sibs studied that is to say those who were HL A identical

We made the analysis of 156 kidney trans plantations performed in Paris Obviously the best ones are those performed between HL A identical sibs All the 24 cases are still in per fect condition (27 31) These data are con verging proofs that the HL A system is the main histocompatibility system in man that the other histocompatibility systems are weak having no activity in MLC test or under im muno-depression However there are other systems undoubtedly since skin grafts are nevertheless rejected between HL-A identical sibs.

The situation is not so good in HL-A haplo identical situation, that is to say when one haplotype is shared by donor and recip ient (grafts performed between HL-A haplo identical sibs or between child and parent) Here the possible presence of incompatibilities governed by the haplotype which is not shared is sufficient to shorten the survival time of either skin or kidney grafts In our statistics 86 per cent of success are counted.

The results are less good in HL A different situation, between unrelated individuals when donors and recipients differ on the two HL A chromosomes Here only 56 per cent of surv ivals are observed. The fact that the results are directly related to the number of HL A haplotypes genetically common to donors and recipients is striking (27 37)

A direct correlation between the number of incompatibilities and the fate of kidneys has also been found The maximum of HL A incompatibilities between two individuals is four (2 loci on two chromosomes) 85 per cent of the grafts with 3 incompatibilities out of 4 were rejected 55 per cent with two incom patibilities 22 per cent with one incompati bility and 6 per cent with no detectable in compatibility

It is now urgent to determine whether some HL A antigens are more immunological than others For this purpose *Felix Rapaport* and I have carried out an experiment of skin grafts performed from children to fathers (25 26) The father was grafted with two skins coming from two children each of them having received one of the two different maternal haplotypes C or D 238 grafts were made We observed that the skins were always rejected in a bimodal fashion For example, the skins bearing the C haplotype were re jected in 10 days and those bearing the D haplotype in 13 to 14 days This also indi cates that the HL A system is the main trans- plantation system for even when they are identical for the HL-A system, sibs may re ceive different sets for the other H systems

The mean survival time of the skin allo grafts for the HL A compatible grafts was 15 10 ± days in contrast with 12 I ± days for the incompatible grafts ($p < 0(1)$

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TABLE 1 *The Injected Material the Dose Schedule and Cycles of Treatment*

Experiment	Number of rabbits	Injected material	Dose schedule	Cycle of treatment
I	10	Freshly prepared homologous heart homogenate	100 mg s c + 100 mg i p × 3 a week	3 × 3 weeks treatment + 2 × 3 weeks interval
	10	Five times frozen (— 30 C) and thawed (+20 C) homologous heart homogenate	100 mg s c + 100 mg i p × 3 a week	3 × 3 weeks treatment + 2 × 3 weeks interval
II	5	Freshly prepared homologous heart homogenate	100 mg s c + 100 mg i p daily	1 week
	5	*Five times frozen (—30 C) and* thawed (+20 C) homologous heart homogenate	*100 mg s c + 100* mg i p daily	1 week

s c = subcutaneously i p ~ intraperitoneally

sequently once a week Eighteen weeks after the beginning of the experiment five of the animals in both groups of Experiment I were killed under Nembutal anaesthesia after blood had been drawn by heart puncture A part of the kidneys of all the animals were kept for immunofluorescence examination and stored at *—20 C Four* in both groups were killed in the same way 14 weeks later Two of the animals died just before the termination of the experiment

Passive Haemagglutination

A tanned cell haemagglutination method described by Boyden and modified by *Gery & Davies* (7) was used with some further modifications Two ways of centrifuging the rabbits heart homogenate were used to prepare the antigens 120 000 g min (moderately centrifuged antigen) and 2 × 6 000 000 g min (soluble antigen) *For the sake* of comparison both freshly prepared homogenates d those stored deep frozen for several weeks u ed in each method The sera w re heated 56 C *for* 30 *minutes* absorbed with tanned cells and diluted in doubling dilution series beginning with 1 to 5

Gel Precipitation

The double diffusion method of Ouchterlony as used Soluble test antigens were prepared as described above

Fluorescent Antibody Technique

Gamma globulin separated from rabbit serum was used to immunize guinea pigs The resulting antiserum was *fractionated with* $(NH_4)_2SO_4$ to yield gamma globulin which was labelled with fluorescein isothiocyanate (provided by Baltimore Biological Laboratories) as described by *Holborow & Johnson* (10)

Tissue sections 6 microns thick were cut in a cryostat at —20 C transferred to glass slide and air dried at room temperature After washing in phosphate buffered saline pH 7 2 for 30 min under constant agitation they were fixed in 95 per cent ethanol for 10 min The sections were *incubated in a moist chamber with one drop of* fluorescein conjugated antirabbit globulin for 30 min at room temperature and washed again for one hour A Leitz fluorescence microscope with an O ram HBO mercury lamp was used for the reading of the results

RESULTS

Tissue Damage

*The kidney lesions produced by heart ho*mogenate injections in the long term experiment (Experiment I in *Table 1*) are described in the first paper (14) The findings *were interpreted* as an experimental model of chronic tubulo interstitial nephritis Comparison of the incidence of lesions and their severity showed that the animals that were *given freshly prepared* homogenate exhibited in higher frequency a *significantly more ad*vanced disease than animals injected with denatured—frozen and thawed—heart ma*terial*

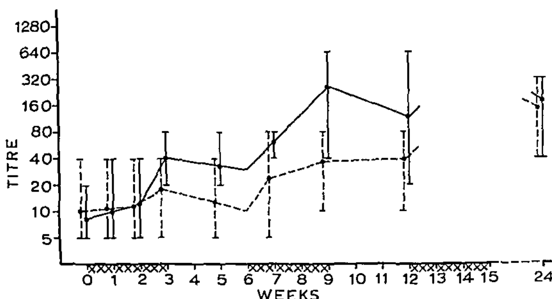


Fig 1 Passive haemagglutination activity against moderately centrifuged (120 000 g min) homologous heart homogenate The curve is drawn through the average titre at each interval

———— rabbits injected with denatured heart homogenate
------- rabbits injected with freshly prepared heart homogenate
XXXX X injection period

Passive Haemagglutination

No essential difference between the titres obtained with freshly prepared heart antigens and those stored deep frozen for a few weeks was noted The freshly prepared antigen however especially when centrifuged at less than 120 000 g min easily caused an unspecific aggregation of cells For these reasons it was decided to use only antigens prepared from hearts stored deep frozen for a few weeks

Untreated animals When moderately centrifuged (120 000 g min) antigen was used anti heart antibodies in titres up to 40 were found in the sera of untreated animals (Fig 1 and Table 2) These natural antibodies were not destroyed by heating the sera to 56 for 30 min When soluble antigen was used for the sensitization of red cells no activity was noted in normal sera (Fig 2) Natural anti heart antibodies therefore seem to react against particulate tissue components only

The injected animals The changes in the titres of anti tissue antibodies tested with moderately centrifuged antigen during the main experiment are presented in Fig 1 The main characteristic is that the denatured heart homogenate was a stronger immunogen

TABLE 2 *Passive Haemagglutination Activity against Moderately Centrifuged Homologous Heart Homogenate in Sera of Rabbits Injected daily with Homologous Heart Homogenate*

Rabbit	Titre before treatment	Titre after 7 days of treatment
D 1	5	160
D 2	20	160
D 3	5	80
D 4	10	320
D 5	20	320
F 1	20	40
F 2	10	40
F 3	10	10
F 4	10	20
F 5	10	20

D = injected with denatured heart homogenate
F = injected with freshly prepared heart homogenate

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DISTRIBUTION OF THE ABO, MNS, P, RHESUS, LUTHERAN, KELL, LEWIS AND DUFFY BLOOD GROUPS AND FREQUENCY OF IRREGULAR RED CELL ANTIBODIES IN A POPULATION OF DANES AGED FIFTY YEARS AND A POPULATION OF DANES AGED SEVENTY YEARS

From the Glostrup Population Studies

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The blood group and gene frequencies within the ABO MNS P Rhesus Lutheran Kell Lewis and Duffy systems have been determined in samples from 802 Danes aged 50 years and 427 Danes aged 70 years living in a suburban area of Copenhagen An antibody screening test was performed on sera from the same persons When the results of the blood groupings in the two materials were compared no significant difference could be demonstrated When the present materials were compared with those reported earlier for Danish populations no significant difference was observed The frequency of irregular antibodies against erythrocytes in the two materials was lower than that in materials of patients in Rigshospitalet but the difference was not significant

The variation in blood groups and gene frequencies in Denmark have been studied mainly in blood donors conscripts and parties involved in paternity cases Blood donors are often selected on the basis of their blood types (both ABO and Rhesus) and both conscripts as well as parties involved in paternity cases are males. In Denmark regional variations in blood group frequencies have been demonstrated by *Ernst Goldschmidt* (1) and *Henning Pedersen* (9)

In the present study people were selected on the basis of their age and their place of residence Blood samples from persons born in 1897 and 1914 and living in a suburban

Received 9 iv 70

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TABLE 1 *Blood Group and Gene Frequencies within the ABO System (Expected Number in Brackets)*

Investigation		50 years	70 years	Cutler (2)
Number investigated		802	427	12123
Number	A_1	255 (253 9)	134 (135 1)	4091
	A	92 (91 7)	47 (48 3)	1261
	B	85 (89 4)	52 (47 6)	1245
	O	329 (330 2)	177 (175 8)	5046
	A_1B	35 } (37 9)	13 } (20 1)	341
	A B	6	4	139
Proportion	A_1	0 3180	0 3138	0 33 5
	A	0 1147	0 1101	0 1040
	B	0 1060	0 1218	0 1027
	O	0 4102	0 4145	0 4162
	A_1B	0 0436	0 0304	0 0281
	A B	0 0075	0 0093	0 0115
Gene frequencies	A_1	0 2005	0 1906	0 2056
	A	0 0805	0 0776	0 0745
	B	0 0817	0 0845	0 0741
	O	0 6373	0 6473	0 6458
$\chi = \frac{(o-e)^2}{e}$	A_1	0 0052	0 0097	
	A	0 0183	0 0345	
	B	0 2166	0 4067	
	O	0 0044	0 0082	
	AB	0 2622	0 4924	
0 80 < p < 0 90	total	1 4582	4 d f	

area of Copenhagen have been examined The main purpose of these studies was, first of all, to investigate the state of health but in addition blood samples were sent in for blood grouping in the Blood Bank and Blood Grouping Department University Hospital It has been possible to analyse eight blood group systems in the two materials At the same time an antibody screening test for irregular antibodies against erythrocytes was performed on all sera from the persons investigated The findings have been compared with those obtained in some other Danish investigations A similar study on randomly selected Swedish men aged 50 years has been made in Gothenburg (8)

MATERIALS AND METHODS

During 1964 a population study of people born in 1914 was performed at the County Hospital of Copenhagen in Glostrup The response rate was 85 per cent and the material contains 436 men and 366 women (3)

During 1967 a population study of people born in 1897 was performed at the County Hospital of Copenhagen in Glostrup The response rate was 66 per cent and the material contains 222 men and 205 women

Serological Techniques

Routine Blood Grouping ABO and Rhesus D grouping was performed with test sera of different origin using proper positive and negative controls Reversal grouping was performed with A_1 and B test cells All Rhesus D negative samples were tested with Dextran® fortified anti CD and anti E from the State Serum Institute Copenhagen and all positive samples were tested for the factor D^u with two specially selected incomplete anti D sera using anti globulin technique

An antibody screening test was performed on all sera from the persons investigated using five different cell suspensions with antigens covering all commonly occurring antibodies using saline technique at room temperature saline technique at 37 C followed by indirect Coombs test 2 step papain technique at 37 C and trypsin technique followed by indirect Coombs test Identification of antibodies found in the screening test was performed with a selected panel of cells

TABLE 2 *Blood Group and Gene Frequencies within the MNS System (Expected Number in Brackets)*

Investigation		50 years	70 years	Gurtler (2)
Number investigated		795	427	12123(5115)
Number	MM	216 (219.9)	122 (118.1)	3447
	NN	188 (186.1)	98 (99.9)	6089
	MN	391 (389.0)	207 (209.0)	2587
	MMS	*143 (144.4)*	*79 (77.6)*	*964*
	MMs	73 (75.5)	45 (40.5)	484
	NNS	52 (46.8)	20 (25.2)	322
	NNs	*136 (139.2)*	*78 (74.8)*	792
	MNS	194 (195.8)	107 (105.2)	1282
	MNs	197 (193.2)	100 (103.8)	1271
Proportion	MM	0.2717	0.2857	0.2865
	NN	0.2365	0.2295	0.2160
	MN	0.4918	0.4848	0.4975
	MMS	*0.1799*	*0.1850*	0.1892
	MMs	0.0918	0.1007	0.0973
	NNS	*0.0654*	*0.0468*	*0.0616*
	NNs	0.1711	0.1827	0.1544
	MNS	0.2440	0.2506	0.2523
	MNs	0.2478	0.2341	0.2452
Gene frequencies	M	0.5176	0.5281	0.5353
	N	*0.4874*	*0.4719*	0.4645
	Ms	0.3024	*0.3070*	0.3120
	MS	0.2152	0.2211	0.2233
	Ns	0.4124	*0.4124*	*0.3927*
	Nb	0.0700	0.0595	0.0718

$x^2 = 2.3718$ $0.30 < p < 0.40$ f 2 d f

Genotyping All test sera were used with proper positive and negative controls

A_1A_2 Anti A_1 (bean) from the State Serum Institute slide technique
Anti H (chicken) from the State Serum Institute slide technique

MNS Anti M and anti N (rabbit) from the State Serum Institute slide technique
Anti S from Rigshospitalet in direct Coombs technique

Rhesus Anti C+C^w from Rigshospitalet saline technique
Anti E from the State Serum Institute saline technique
Anti c (two) from Rigshospitalet in direct Coombs technique
Anti e commercially available tech

TABLE 3 *Blood Group and Gene Frequencies with the P System*

Investigation		50 years	70 years	Henningsen (4)
Number investigated		798	427	2345
Number	P_1	595	338	1849
	P_2	203	83	496
Proportion	P_1	0.7456	0.7916	0.7885
	P_2	0.2543	0.2084	0.2115
Gene frequencies	P_1	0.4957	0.5435	0.5401
	P	0.5043	0.4565	0.4599

$x^2 = 3.$[illegible]35 $0.05 < p < 0.10$ 1 d f

TABLE 1 *Blood Group and Gene Frequencies within the ABO System (Expected Number in Brackets)*

Investigation		50 years	70 years	Gurtler (?)
Number investigated		802	427	12123
Number	A_1	255 (253 9)	134 (135 1)	4091
	A	92 (91 7)	47 (48 3)	1261
	B	85 (89 4)	52 (47 6)	1245
	O	329 (330 2)	177 (175 8)	5046
	A_1B	35 } (37 9)	13 } (20 1)	341
	A_2B	6	4	139
Proportion	A_1	0 3180	0 3138	0 3375
	A	0 1147	0 1101	0 1040
	B	0 1060	0 1218	0 1027
	O	0 4102	0 4145	0 4162
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An antibody screening test was performed on all sera from the persons investigated using five different cell suspensions with antigens covering all commonly occurring antibodies using *saline technique* at room temperature, saline technique at 37 C followed by indirect Coombs test, papain technique at 37 C and trypsin technique followed by indirect Coombs test. Identification of antibodies found in the screening test was performed with a selected panel of cells.

TABLE 8 *Blood Group and Gene Frequencies within the DUFFY System*

Investigation		50 years	70 years	Gurtler (2)
Number investigated		798	4[illegible]7	[illegible]36
Number	Fy (a+)	516	288	1813
	Fy (a−)	282	139	[illegible]
Proportion	Fy (a+)	0 6466	0 6[illegible]45	0 6[illegible]5
	Fy (a−)	0 3533	0 3255	0 3[illegible]4
Gene frequencies	Fy	0 435	0 433	0 4[illegible]
	Fy^b	0 565	0 567	
estimated	Fy	0 03	0 03	
$\chi^2 = 1\ 045$	$0\ 30 < p < 0\ 40$	1 d f		

TABLE 9 *Antibodies Detected in the Antibody Screening Test*

Material	50 years	70 years	Patients Rigshospitalet 1964	Patients Rigshospitalet 1967	[illegible]
Number investigated	802	427	19 879	21 925	[illegible]
Immune antibodies per cent*	0 8	0 9	1 2	1 4	
Antibodies total per cent	1 [illegible]	1 7	1 9	3 2	1

* Rhesus Kell Duffy Kidd

within several blood group systems does not change with age Nothing in this investigation points in the direction that the surviving part of a population has a gene frequency within the blood group systems investigated that differs from that of the population as a whole

The results of the antibody screening tests are summarized in Table 9 For comparison, some investigations from Rigshospitalet are included in the table The frequency of irregular antibodies against erythrocytes in the population aged 50 years and the population aged 70 years is lower than that in the very selected material of patients from Rigshospitalet, but the difference is not significant

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EFFECT OF CENTRIFUGATION ON THE IMMUNE REACTION OF RABBIT BLOOD LYMPHOCYTES FOLLOWING ANTIGEN STIMULATION *IN VITRO*

J O Lamvik and T Godal

The Gade Institute Department of Pathology University of Bergen Norway

An increase in the immune reaction towards sheep red cells (SRC) was observed in SRC stimulated cultures containing leucocytes from SRC immunized rabbits when the leucocyte SRC suspensions were centrifuged at high speed before incubation Immune reaction was also evident in a majority of culture tubes containing purified lymphocytes stimulated with SRC and centrifuged at high speed while no response was observed in non centrifuged tubes The findings indicate that SRC antigen may stimulate lymphocytes directly without the presence of phagocytes if a close contact is obtained between antigen and reacting cells

Lymphocytes may be separated from mononuclear phagocytes by various methods (*Ling* 1968) Purified lymphocytes from sensitized animals have shown a reduced capacity to respond *in vitro* to antigenic stimulation with blastoid transformation and antibody production (*Oppenheim et al* 1966 *Lamvik* 1969) This observation may be explained in two ways 1 Mononuclear phagocytes participate in the immune response in vitro When these cells are eliminated sensitized lymphocytes will not be stimulated by the antigen 2 Lymphocytes are damaged by the procedure used for purification of these cells making them unable to participate in an immune reaction

In a previous report (*Lamvik* 1969) the *in vitro* immune response of primed leucocytes from rabbits was found to be positively correlated to the number of mononuclear phagocytes present in the leucocyte suspensions used for culturing, whether or not the cells had passed through cotton wool columns This finding would favour the first possibility

Further evidence against the last possibility was achieved in the present study where we found that centrifugation of the leucocyte antigen mixtures enhanced the immune response *in vitro* Furthermore the presence of phagocytes was not found to be an indispensable prerequisite for the immune response towards sheep red cells (SRC) since purified lymphocyte cultures were found capable of responding to SRC *in vitro* after high speed centrifugation

MATERIALS AND METHODS

Leucocyte Cultures

Leucocytes from SRC immunized and non immunized rabbits were obtained by heart puncture followed by defibrination and sedimentation of the erythrocytes in 3 per cent gelatine in saline The immunized rabbits were bled from 2 to 30 weeks

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after the last immunizing dose The leucocyte suspensions from 10 immunized and 4 non immunized rabbits contained on the average 35 per cent granulocytes (SD 8 6) and 2 per cent monocytes (SD 0 8) without significant differences between the suspensions from immunized and non immunized rabbits

Leucocyte cultures were prepared in tissue culture medium (TC 199) with 20 per cent pooled normal rabbit serum as previously described (*Lamvik* 1968a) The cultures containing ? 5 × 10 leucocytes in 2 5 ml of medium per tube were stimulated by the addition of 0 5 ml of TC 199 containing 1 per cent SRC (5 × 10 sheep red cells) while 0 5 ml of TC 199 without SRC was added to the non antigen stimulated controls The pooled normal rabbit serum used in the culture medium contained complement and anti SRC lytic antibodies in low titres (4–8 in the final medium) while anti SRC agglutinins were found in trace amounts in the serum The final medium *showed no agglutination of SRC*

Seven cell rich leucocyte suspensions 5 obtained from SRC immunized rabbits and 2 from non immunized ones were divided in two parts One part of each suspension was handled as described above The lymphocytes in the other part were separated from phagocytic cells by incubation in and filtration through cotton wool columns (*Lamvik* 1966) The leucocyte suspensions before filtration contained ?9 ?5 per cent granulocytes (SD 4 7) and 1 75 per cent monocytes (SD 0 9) while the eluates from the columns contained 2 8 per cent granulocytes (SD 1 1) and 0 35 per cent monocytes (SD 0 3)

The cells in the eluates from the columns were washed in TC 199 and suspended in culture medium and the cell counts corrected to the same cell concentration as in the non filtered cell suspensions These lymphocyte cultures were prepared and stimulated as described above

The culture tubes were divided into three groups before start of incubation Each group usually contained duplicate culture tubes with cells from each cell suspension One group was incubated without centrifugation The tubes in the other group were centrifuged at 800 revolutions per min (150 g) at room temperature for 5 min while the tubes in the third group were centrifuged at 3 200 revolutions per min (2 100 g) for 5 min

Some culture tubes were harvested before incubation and the vitality of the cells evaluated by testing their ability to exclude eosin according to *S h k* (1936) The other tubes were incubated at 37 C with a change of medium after 3 days and harvested after 7 days Due to the lytic antibodies present in the normal rabbit serum used in the culture media the stimulating SRC added in excess at the start were lysed completely within ?4 hours Therefore the stimulating SRC did not interfere with the subsequent test for plaque forming cells

Testing for Immune Reaction

At the time of harvesting the cultured cells were washed in TC 199 suspended in 0 4 ml of cold TC 199 and tested for plaque forming ability against SRC in micro incubation chambers prepared with double sticky tape according to *Cunningham & S enberg* (1968) The cells were mixed in equal volumes with TC 199 containing 8 per cent SRC and ?0 per cent fresh pooled SRC adsorbed guineapig serum as source of complement before transfer to the incubation chambers The chambers were incubated at 37 C for 30–60 min before being examined for lytic plaques using phase contrast microscopy The mean numbers of plaque forming cells in duplicate incubation chambers were estimated Cell count and differential count were carried out in incubation chambers following the addition of acridine orange stain (*Lamvik* 1968b) The numbers of plaque forming cells as well as the cell counts and differential counts were determined in blind labelled incubation chambers

The cell numbers found in the incubation chambers depended on the cell numbers in the culture tubes since a constant part (about 2 per cent) of the harvested cell suspensions was plated in each chamber The absolute number of plaque forming cells at harvesting was used as measure of immune response since total cell number as well as numbers of plaque forming cells both depended on the antigen induced cell proliferation in the culture tubes

Anti SRC agglutinin titres were determined in the culture media at the time of medium change and at harvesting

RESULTS

On vital staining of the cells in culture tubes with and without centrifugation but prior to incubation less than 5 per cent of the cells in the leucocyte cultures took up the stain granulocytes mainly being stained while the number of stained cells in the cultures containing purified lymphocytes was less than 1 per cent *There was no difference between* centrifuged and non centrifuged cultures

The cellular and specific immune response observed in 10 SRC stimulated leucocyte culture series containing cells from SRC immunized rabbits are shown in Figs 1 and 2 The response as measured by cell counts and blastoid cells and by numbers of plaque

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after an interval of 3–4 h At this time the leucocytes could be less able to respond than at culture start In the centrifuged cultures the antigen may be brought rapidly in contact with functionally more active phagocytes and lymphocytes thereby explaining the increased immune response However it is not our experience that the period of time (3–6 hours) from rabbit bleeding to centrifugation of the leucocyte SRC mixture is an important factor for the immune response *in vitro* Furthermore this time factor can hardly explain the difference in response between cultures centrifuged at 800 rpm and 3 200 rpm

In non centrifuged cultures phagocytic cells seem to be of importance since elimination of such cells by cotton wool filtration gives cell suspensions without definite reactivity towards SRC A similar dependence on phagocytic cells has been observed for a number of other antigens (*Campbell & Garvey* 1963 *Makinodan & Albright* 1966) However the dependence of mononuclear phagocytes seems to vary from one antigen to the other (*Boak et al* 1969) Some antigens such as keyhole limpet haemocyanin apparently may elicit immune response in the absence of macrophages by triggering the lymphocytes directly

In our view the most likely explanation of the present findings is that SRC may be able to trigger the lymphocytes directly when SRC and lymphocytes are packed together by centrifugation Thus the present study may support the hypothesis that the role of mononuclear phagocytes in immune response is to facilitate the reaction between the antigen and specific receptor sites on lymphocytes A breakdown or digestion of the antigen by the phagocytes is apparently not a requisite for this function

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PULSE-RNA-DNA HYBRIDIZATION BETWEEN RODSHAPED AND COCCAL SPECIES OF THE *MORAXELLA-NEISSERIA* GROUPS

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Pulse RNA H extracted from 4 species of moraxellae and neisseriae were hybridized to DNA from 14 species of rodshaped and coccal organisms belonging to these groups one species of *Acinetobacter* and from *Escherichia coli* in 44 out of 64 possible combinations The affinities observed ranged from zero to 34 ? per cent of autologous hybridization activity (DNA and RNA from the same strain) In the combinations of strains studied the hybridization affinities were essentially of the same relative order as in transformation of streptomycin resistance Additional evidence was provided in favour of a recent redefinition of the genera *Moraxella* and *Neisseria* in which each genus contains both rodshaped and coccal organisms

None of the criteria used as a basis for discussion of bacterial classification at the molecular level can probably be applied as the sole yard stick for taxonomic relations which makes a combination of methods desirable Thus both genetic transformation with streptomycin resistance as the genetic marker and determination of average DNA base composition have previously been used for the elucidation of relationship between organisms of the *Moraxella Neisseria* groups (3 5 6 7 8 9 10 12 13 14 18 19) One also considered the application of in vitro nucleic acid hybridization in the same material of species and strains

DNA DNA hybridization which was used by *Kingsbury (23)* and *Kingsbury et al* (24) in studies on relations between *Neisseria* species is a technically demanding procedure It is known also from other studies that differences in methodological stringency in relation to the thermal stabilities of the DNA DNA hybrids and other factors may lead to dissimilar estimates of homology (22 26) Exploration of pulse RNA DNA hybridization in the segmental study of bacteriophage transcription showed the degree of regional specificity and reproducibility of hybrid formation under different conditions (11), and a modification of the latter method was chosen as a potentially equivalent or better alternative for demonstration of DNA homologies and heterologies in these bacteria with respect to the transcribed regions

A question of methodological importance

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was whether the pattern of compatibilities in pulse RNA DNA hybridization would closely follow that observed in streptomycin resistance transformation One mainly wanted to test the validity of previous conclusions on gross relations between rodshaped and coccal species, i e whether it could be confirmed that the genus *Moraxella* and the genus *Neisseria* are sharply distinguishable only after taxonomic revisions which imply that each genus contains rodshaped as well as coccal organisms (10 19)

MATERIALS AND METHODS

Organisms

The majority of the strains studied had been characterized in intraspecies and interspecies genetic transformation (5 6 8 9 10 18) DNA base composition had also been determined in all strains except one (7 10) The base composition of *Neisseria flava* ATCC 14221 DNA was determined according to methods described (7) The designations of the strains the interspecies affinities in streptomycin resistance transformation and the guanine + cytosine (G + C) contents of their DNAs are given in Tables 1 and 2

Media and Solutions

Tryptone broth 1 per cent Tryptone (Difco) 0 5 per cent NaCl 0 001 M MgCl 0 0005 M $CaCl_2$ pH = 7 4

M9b buffer 3 g KH_2PO_4 7 g Na_2HPO_4 1 g NH_4Cl 0 5 g NaCl 1 ml of 1 M $MgSO_4$ 10 ml of 0 01 M CaCl 1 liter of distilled water pH = 7 2

TES buffer 0 15 M NaCl 1 mM Tris 0 01 M EDTA pH = 7 7

SSC 0 15 M NaCl 0 015 M Na_3 citrate pH = 7 4

2 × SSC 6 × SSC 10 × SSC concentration multiples of SSC

Phenol commercial liquefied phenol (A R) distilled and stored in tightly closed 20 ml vials at —20 C or Phenol z Analyse (E Merck A G)

Hybridization fluid 1 volume of 2 × SSC + 1 volume of phenol saturated 2 × SSC prepared just before use and adjusted to pH = 7 4–7 5

pRNase stock solution of 1 mg/ml of pancreatic RNase (code RASE or RAF Worthington Biochemical Corp Freehold N J) in distilled water preheated at 97 C for 10 minutes

T_1RNase stock solution of 500 units/ml of T_1RNase (Worthington Biochemical Corp Freehold N J) in distilled water preheated at 97 C for 10 minutes

Pronase stock solution of 1 mg/ml of pronase (Calbiochem Co Los Angeles Cal) in distilled water selfdigested by 2 hours incubation at 37 C

Labelling and Isolation of RNA ³H

1) A culture of the bacterial strain was grown in Tryptone broth (20 ml in a 125 ml Erlenmeyer flask) under vigorous agitation in an orbital water bath shaker, at 37 C for the strains of *Neisseria catarrhalis N ovis* and *N elongata* and at 33 C for *Moraxella nonliquefaciens* 2) At O D = 0 35–0 40 corresponding to approximately 5 × 10⁸ colonyforming units per ml (see Fig 1 for stage of growth *M nonliquefaciens* grew very slowly without reaching a stationary stage within 15 hours) 0 5 ml of uridine-5 ³H (= 0 5 mCi) was added to the vigorously shaken culture 3) At the end of the pulse labelling (one minute) the 20 ml culture was poured into another 125 ml flask which

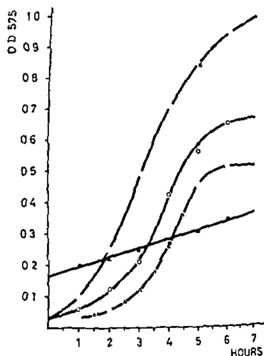


Fig 1 Growth in Tryptone broth (1 per cent Tryptone (Difco) 0 5 per cent NaCl 0 001 M MgCl 0 0005 M CaCl pH = 7 4) of the strains used as donors of pulse RNA ³H

—— *Neisseria catarrhalis* Ne 11 at 37 C.
○——○ *N ovis* 199 55 at 37 C
△——△ *N elongata* M2 at 37 C
●——● *Moraxella nonliquefaciens* 4663/62 at 33 C

All cultures were incubated under constant agitation in an orbital water bath shaker

had been frozen beforehand at —60 C containing 5 ml of prefrozen crushed M9b buffer (supplemented with 0 02 M NaN_3) The mixture was shaken well in an ice water bath until melting and was then transferred to a 30 ml centrifuge tube which was kept cold 4) The cells were spun down at 6000 rpm for 10 minutes at 4 C and the pellet resuspended in 0 5 ml of TES buffer The suspension was transferred to a 15 ml centrifuge tube (Corex) and another 0 5 ml of TES buffer containing 2 5 per cent of sodium dodecyl sulphate was added to give a final concentration of 1 25 per cent of the detergent in a total volume of 1 ml 5) The suspension was heated for 1 3 minutes in a 97 C waterbath followed by cooling at room temperature for 5 minutes 6) The RNA containing solution was thereafter exposed to three successive hot phenol extractions (60–64 C) adjusted to the salt concentration of 2 × SSC by adding 10 × SSC and measured for radioactivity in TCA (trichloroacetic acid) precipitated samples as described by Bøvre & Szybalski (Section II B steps 6–9 of ref 11)

Isolation of DNA

DNA was isolated according to the method of Marmur (27) with some modifications Nucleic acids were extracted as for transformation purposes (4) and the DNA was further purified as follows 1) To 3 ml of the crude extract (200–400 µg of DNA per ml) in a 15 ml centrifuge tube 150 µg of pRNase and 30 units of T_1RNase were added After a 10 second swirl on a Vortex mixer the RNA was digested for 30 minutes at 37 C 2) Following cooling in ice water deproteinization was repeated by adding 1 volume of chloroform isoamylalcohol mixture (24 1 v/v) and manual shaking of the silicone stoppered glass tube at room temperature for 15 minutes The mixture was thereafter centrifuged (10 000 rpm 4 C 10 minutes) 3) The upper phase was slowly pipetted into a small beaker then a ⅓ volume of 3 M sodium acetate was added followed by a dropwise addition of a 0 6 volume of isopropanol while the beaker was shaken by the Vortex mixer The DNA precipitate was wound up on a bent glass rod as soon as it formed and dissolved in 3 ml cold TES buffer 4) After complete dissolution of the DNA (in refrigerator overnight) pronase was added to a final concentration of 50 µg/ml and incubation performed at 37 C for 30 minutes 5) After cooling in ice water 1 volume of water saturated phenol was added and the two phase solution gently mixed at room temperature for one minute followed by centrifugation (10 000 rpm 4 C 10 minutes) The aqueous upper layer was carefully removed and exposed to repeated cycles of phenol treatment until clear and virtually no protein collected at the interphase 6) The last supernatant which was highly viscous was slowly transferred into preboiled dialysis bags dialysis was undertaken against several changes of 1 mM EDTA pH = 7 4 for 36 hours and the resulting DNA solution was stored frozen at —20 C

RNA DNA Hybridization

A *Preparation of filters with denatured DNA* The required amount of DNA (5 µg per filter) was calculated from O D 260 measurements and dissolved in 2 ml of 1 mM EDTA pH = 8 0 and heated at 97 C for 10 minutes Thereafter the denatured DNA was chilled in ice water and kept in the cold The efficiency of denaturation was monitored by observing the rise in optical density The O D 260 was found to be in the range from 134 0 to 136 8 per cent of the values found before denaturation The 2 ml DNA portions were adjusted to the salt concentration of 6 × SSC and loaded by slow filtration on membrane filters (Bac T Flex type B6 24 mm diameter Schleicher & Schuell Keene N H) and the loaded filters were washed with 6 × SSC dried and baked at 80 C all as previously described (Section II E, 1 b steps 2–4 of ref 11) Between 92 and 100 per cent of the denatured DNA was retained by the filters as determined by O D 260 measurements in the filtrates in special experiments with 50 µg of DNA per filter In some cases over 80 per cent of the hybridization capacity of the filterbound DNA was retained after 8 months of storage at room temperature In the experiments to be reported however the DNA filters were usually not more than 1–2 months old and of equally aged batches in the same experimental series

B *Hybridization procedure* The methodology of Gillespie & Spiegelman was generally followed (16) A precalculated volume of RNA ^3H was added to each 5 µg DNA filter in a glass vial (diameter slightly larger than that of the filter) and phanalized 2 × SSC (hybridization fluid) added up to a total of 1 ml in each case where direct comparisons were made between hybridization activities of different DNAs all parallels were prepared with the same input TCA precipitable RNA ^3H counts and incubated under identical conditions annealing took place at 65–67 C for 22 hours The vials were then chilled in ice water Each filter was removed from the vial rinsed on both sides with 10 ml of 2 × SSC mounted for filtration and washed at room temperature with 50 ml of 2 × SSC passed through each side of the filter under high vacuum. The filter was then transferred to a vial with 2 ml of 2 × SSC supplemented with 40 µg of pRNase and 20 units of T_1RNase incubation took place at room temperature for 1 hour All the vials of a series were then chilled and kept in ice water until rinsing and washing with 2 × SSC could be repeated (see above) Finally the f[illegible]

was whether the pattern of compatibilities in pulse RNA DNA hybridization would closely follow that observed in streptomycin resistance transformation One mainly wanted to test the validity of previous conclusions on gross relations between rodshaped and coccal species, *i e* whether it could be confirmed that the genus *Moraxella* and the genus *Neisseria* are sharply distinguishable only after taxonomic revisions which imply that each genus contains rodshaped as well as coccal organisms (10 19)

MATERIALS AND METHODS

Organisms

The majority of the strains studied had been characterized in intraspecies and interspecies genetic transformation (5 6 8 9 10 18) DNA base composition had also been determined in all strains except one (7 10) The base composition of *Neisseria flava* ATCC 14221 DNA was determined according to methods described (7) The designations of the strains the interspecies affinities in streptomycin resistance transformation and the guanine + cytosine (G + C) contents of their DNAs are given in Tables 1 and 2

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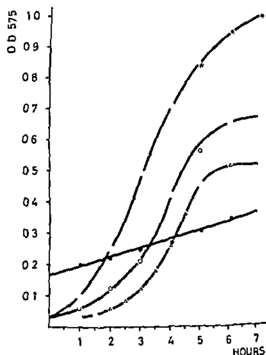


Fig 1 Growth in Tryptone broth (1 per cent Tryptone (Difco) 0 5 per cent NaCl 0 001 M MgCl 0 0005 M CaCl pH = 7 4) of the strains used as donors of pulse RNA ³H

—— *Neisseria catarrhalis* Ne 11 at 37° C
O——O *N ovis* 199/55 at 37 C
Δ——Δ *N elongata* M2 at 37 C
●——● *Moraxella nonliquefaciens* 4663/62 at 33 C

All cultures were incubated under constant agitation in an orbital water bath shaker

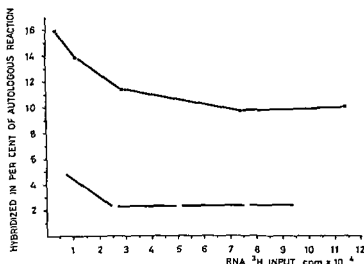


Fig 4 Heterologous hybridizations between *Neisseria ovis* 199/55 pulse RNA ^{3}H and 5 µg DNA of *Moraxella bovis* ATCC 10900 (●——●) and *N catarrhalis* Ne 11 (▲——▲) in per cent of autologous *N ovis* 199/55 RNA DNA hybridization at different inputs of TCA precipitable RNA H The values (cpm) for the heterologous hybridizations can be read from Fig 3 and those for the autologous hybridizations can be found in Fig 2

complement than does for instance rRNA (ribosomal RNA) (28) There is virtually no hybridization in simultaneously observed controls with *Escherichia coli* DNA (Fig 3)

The amounts of *N ovis* pulse RNA ^{3}H forming hybrid with *M bovis* and *N catarrhalis* DNAs (Fig 3) as percentages of the autologous *N ovis* RNA DNA hybridization (Fig 2) are expressed in Fig 4 At all levels of RNA input the homologies of RNA from the coccal *N ovis* appear more pronounced with DNA from the rodshaped *M bovis* than with DNA extracted from the coccal *N catarrhalis* At the lowest RNA inputs tested the heterologous hybridizations are relatively high amounting to 16 per cent of the autologous *N ovis* reaction in the case of *M bovis* DNA and to 5 per cent with *N catarrhalis* DNA At higher RNA inputs however the relative values of the heterologous reactions at first decrease and then level off at about 10 per cent and 2 5 per cent of the autologous hybridization respectively To avoid possibly non representative phenomena and to secure methodological stability RNA ^{3}H inputs in the plateau region (Fig 4) were chosen as standard in comparative experiments (approximately 75 000 cpm for the RNA preparations of *M nonliquefaciens* *N catarrhalis* and *N ovis* and 28 000 cpm for the RNA extracted from *N elongata*) The inputs chosen (cpm TCA precipitable counts corresponded to $^1/_{15}$–$^1/_{10}$ of the label recovered in RNA from 10^{10} cells Usually 10–15 per cent and occasionally up to 19 per cent of these inputs would form RNase resistant hybrid with 5 µg of autologous DNA The latter efficiencies are typical of mRNA DNA hybridizations (15, 28) However it was obvious from comparisons with *E coli* under similar but not identical conditions that the yield of uridine 5 ^{3}H labelled RNA was low in all the organisms ($^1/_{10}$ or less of the incorporation in *E coli* RNA) especially in the case of *N elongata* In this organism the amount of label taken up or fixed per minute (retained by the cells after washing), was very low but distinct and constant during a 5 minutes test period The short pulse (one minute) was maintained to keep the background rRNA activity at a minimum The choice of a usually specific RNA labelling compound was a safety measure against the possible labelling of DNA and its eventual activity in the hybridization system That this possibility is remote was shown by treating one of the RNA extracts of *M nonliquefaciens* with pRNase and T$_1$RNase before testing it in autologous hybridization No trace of hybridization activity remained Also the results obtained after low temperature cell lysis DNase treatment and the ... backgr

TABLE 1 *Pulse RNA DNA Homologies of Representati e* Moraxella *and* Neisseria *Strains as Compa ed with DN 4 Base Composition and Transformation Affinities*

DNA[a]		Affinity in per cent of autologous reaction[b]					
Source[c]	(G+C) content[d]	Genetic transformation[e] (recipient)			RNA DNA hybridization[f] (source of RNA)		
		M nonliq 4663/62	*N catarrh* Ne 11	*N ovis* 199/55	*M nonliq* 4663/62	*N catarrh* Ne 11	*N ovis* 199/55
M nonliquefaciens 4663/62	42 0	100	0 007	0 01	100	8 4	4 9
M liquefaciens NCTC 7911	41 5	0 4	0 005	0 02	34 2	4 6	8 7
M lacunata ATCC 11748	42 0	0 4			22 2	4 5	7 5
M bovis ATCC 10900	42 5	0 2	0 004	0 08	13 1	2 4	9 7
N catarrhalis Ne 11	41 0	+	100	0 004	6 5	100	2 4
N caviae ATCC 14659	41 5		0 001	0 05	4 5	2 5	5 5
N ovis 199/55	45 0	+	0 001	100	7 1	2 9	100
M osloensis A 1920	43 5	+	0 002	0 002	2 0		
M phenylpyrouvica 2863	43 0	0	0 0005	0 001	1 2		
A lwoffi ATCC 17985	43 0	0	(+)	(+)	0 6		
M kingii 4177/66	44 5	0	0	0	0 4		
N cinerea 165/61	49 0		0	0	0 6	0 7	0 6
N flavescens ATCC 13120	47 5	0	0	0	0 3	0 6	0 7
N flava ATCC 14221	47 5	0			0 8	0 5	0 5
N elongata M2	53 0	0	0	0	0 5	0 00	0 [illegible]
E coli W3350	(50 0)				0 00	0 01	0 14

a In transformation donor DNA (sometimes replaced by DNA of another strain of the same species) in hybridization filterbound denatured DNA b Autologous = intrastrain reaction recipient transformed by DNA extracted from a mutant of the same strain or hybridization between RNA ³H and DNA of the same strain c Strains characterized in previous studies (5 6 8 9 10 18) *M* = *Moraxella* *N* = *Neisseria* *A* = *Acinetobacter* *E* = *Escherichia* d All strains except *N flava* ATCC 14221 previously determined (7 10) *E coli* DNA was assumed to contain 50 per cent (G + C) e Streptomycin resistance transformation (5 6 9 10 18) In the case of *M nonliquefaciens* the strain NCTC 7784 was often used as recipient + = low affinity more distinct in the reciprocal reaction (+) = very few transformants observed f Cultures were grown to O D $_{75}$ = 0 35–0 40 in Tryptone broth RNA was then pulse labelled for 1 minute with uridine 5 ³H (see Fig 1 for stage of growth at labelling) and isolated by hot phenol extraction 75 000 cpm of TCA precipitable RNA ³H from each of the three strains was simultaneously hybridized to 5 μg of filter bound denatured DNA of the autologous and heterologous strains (column of DNA source) See legend of Fig 2 for outline of hybridization procedure

values observed with *E coli* DNA and blank filters (see below) do not indicate that denatured DNA (labelled or unlabelled) or protein in the RNA extracts played a significant role in the hybridizations

In Table 1 are listed the interspecies hybridization reactions observed between *M nonliquefaciens* *N catarrhalis* and *N ovis* RNAs and DNAs from 14 species of oxidase positive organisms one oxidase negative species (*Acinetobacter lwoffi*) and from *E coli* Among unequivocal moraxellae (*M nonliquefaciens* *M liquefaciens* *M lacunata* and *M bovis*) the heterologous hybridizations amount to 13 1–34 2 per cent of the autologous *M nonliquefaciens* reaction Between these organisms and false neisseriae (*N catarrhalis* *N caviae* and *N ovis*) there are affinities in the range from 2 4 per cent to 9 7 per cent whereas the homologies between the false neisseriae themselves range from 2 4 per cent to 5 5 per cent *M osloensis* and *M phenylpyrouvica* reveal 1 2–2 per cent hybridization compatibilities with *M nonlique*

faciens Further down the scale, *A lwoffi M kingii N cinerea N flavescens N flava* and *N elongata* exhibit heterologous to autologous ratios against *M nonliquefaciens N catarrhalis* and *N ovis* (to the extent they were tested) in the range from zero to 0 8 per cent *E coli* DNA shows either zero or only up to 0 14 per cent such homology with RNA from the oxidase positive organisms

TABLE 2 *Pulse RNA DNA Hybridization and Transformation Compatibilities of* Neisseria elongata *and* N flava *as Compared with the Affinities between* N elongata *and* Moraxella nonliquefaciens

Source of DNA	Affinity in per cent of autologous reaction[b]	
	Genetic transformation (recipient *N elongata* M2)	Hybridization (source of RNA *N elongata* M2)[f]
M nonliquefaciens 4663/62	0	0 4
N flava ATCC 14221	2 1	11 0
N elongata M2	100	100
E coli W3350		0 00

a b c e f See legend to Table 1 Input in hybridization 28 000 cpm TCA precipitable RNA ^{3}H

In Table 2 are shown the hybridization affinity of *N elongata* RNA to *M nonliquefaciens* and *N flava* DNA which is 28 × higher to the latter (11 per cent) than to the former (0 4 per cent) Also with this RNA there is no homology observed with *E coli* DNA

Certain RNA ^{3}H preparations or hybridization experiments revealed relatively pronounced binding to control filters under annealing conditions for unexplored reasons No result from such experiments are included in the figures or the tables where all background values were low (with most sources of RNA 0 02 per cent or less of the input TCA precipitable counts bound to a DNA free filter total range 0 006–0 07 per cent) However imperfect experiments provided supporting evidence, since they gave relative hybridization values consistent with those recorded after subtraction of the background activities before calculation In one such experiment 5 μg of filter bound denatured DNA from *M nonliquefaciens* NCTC 7784 (G + C content 41 per cent transformation affinity to *N catarrhalis* Ne 11 0 003 per cent) was hybridized to 72 000 cpm of *N catarrhalis* Ne 11 RNA, pulse labelled at a comparatively late stage of growth (O D$_{5\,5}$ = 0 90 see Fig 1) The hybridization activity was 8 9 per cent out of simultaneously observed autologous *N catarrhalis* Ne 11 RNA DNA hybridization (15 per cent of the input TCA precipitable RNA ^{3}H was hybridized in the autologous reaction)

Results consistent with the data in Fig 4 and Table 1 for relative affinities between *N catarrhalis* Ne 11 *M bovis* ATCC 10900 and *N ovis* 199/55 were obtained in RNA DNA hybridization with pulse RNA ^{3}H isolated from *N ovis* 199/55 without exposing the lysing cells to higher temperature than 60° C (thus avoiding DNA denaturation) Only slightly different values were observed with *N ovis* 199/55 pulse RNA ^{3}H in a two step hybridization procedure including DNase treatment of the RNA solution before the last step The RNA ^{3}H was first hybridized to autologous *N ovis* DNA followed by treatment of the hybrid with pRNase + T$_1$RNase elimination of RNase activity by iodoacetate elution and DNase treatment of the eluate (11) After heat inactivation of DNase 6300 cpm aliquots were hybridized to 5 μg DNA filters with the following results *N catarrhalis* Ne 11 – *N ovis* 199/55 ratio 4 6 per cent *M bovis* ATCC 10900 – *N ovis* 199/55 ratio 9 4 per cent The autologous hybridization efficiency was 9 per cent in the first step and 13 per cent after DNase treatment

DISCUSSION

Most of the heterospecific affinities observed in the present study may at first appear indicative of little taxonomic relationship espe

cially when compared with some recent DNA DNA hybridization studies (17 20 21, 23, 29) The occasional occurrence of measurements of the same relations by both methods indicate however, that the techniques of DNA DNA hybridization reveal higher values of homology, at least under some conditions For instance *Kingsbury* (23) found that *Neisseria catarrhalis* DNA is 28 per cent homologous with *N flavescens* DNA, as compared with 0 6 per cent in the precent study However, increased incubation temperature resulted in a marked decrease of some other interspecies DNA DNA hybrids tested (24), so that the figures compared are certainly not representative of the difference between the two modes of hybridization

In DNA DNA hybridization the labelled DNA fragments are often longer than the homologous regions and the unmatched nucleotide sequences cannot at present be selectively eliminated from the hybrid after annealing The latter obstacle can be avoided in RNA DNA hybridization by means of RNase However some taxonomic RNA DNA hybridization studies have not included RNase treatment of the hybrids before assay As in DNA DNA hybridization the controls of specificity most often are 1) the relatively low affinity of distantly related DNAs or 2) little binding of the radioactive component in the absence of denatured unlabelled DNA Unfortunately such controls do not tell much about the base to base specificity of more active heterologous reactions in the system It as been shown in bacteriophages and bacteria that even limited nucleic acid homology of two organisms may be enough to raise a heterologous RNA DNA hybridization up to a considerable level when RNase treatment is omitted whereas a DNA free control or an autologous reaction may be almost unaffected by the presence or absence of RNase (11 30)

Affinities even as low as in th 2-6 per cent range seem to be compatible th intrageneric relationships in a pulse l A DNA or mRNA DNA hybridization sy m with ontrolled methodology including the use of RNase Evidence in favour of this has been revealed in genus *Bacillus* (15) Also only 5–11 per cent homology was found in a careful mRNA DNA hybridization study of the phages λ and φ80 which must be considered related both from genetic recombination data and from the molecular organization and function of their genomes (27) Although there exists no proof for its superiority over simple pancreatic RNase treatment the combined use in the present study of pancreatic RNase (which attacks nucleotide sequences next to pyrimidine nucleotides) and T_1RNase (attacking sequences only adjacent to guanylic acid) should ensure that only minimal amounts of noncomplementary structures are being measured as hybrids

Because directly comparable reports are scarce the actual percentages of heterologou out of autologous hybridization are of less interest than the relative magnitudes of these ratios as discussed below

With due attention to the fact that most observations were made with one strain only from each species the present hybridization results can be summarized as follows 1) The oxidase positive rodshaped *Moraxella* ros *liquefaciens* *M liquefaciens* *M lacunata* and *M bovis* (unequivocal moraxellae) have as high or higher affinity to false neisseriae (*N catarrhalis* *N caviae* *N ovis*) as have the latter among themselves 2) *M nonliquefaciens* and false neisseriae show little reactivity with *N cinerea* *N flavescens* and N *flava* (representatives of true neisseriae) 3) *N elongata* a rod haped organism has also low affinity to *M nonliquefaciens* whereas the compatibility with *N flava* is high 4) *M liquefaciens* *M lacunata* and *M bovis* have particularly high affinities to *M nonliquefaciens* 5) The compatibility of *M bovis* with *N ovis* is almost as high as with *M nonliquefaciens* 6) *M osloensis* and *M phenylpyrouvica* have lower affinity to *M nonliquefaciens* but still more than have *Acinetobacter lwoffi* *M kingii* *N cinerea* *N flavescens* *N flava* and N *elongata*

Generally there is consistency between the results of hybridization and genetic transfor

mation (Tables 1 and 2) in almost the same combinations of strains (5, 6 8 9, 10 18) The correspondence between genetic trans formation data and DNA base ratios has been discussed previously (5 7, 10) It is parti cularly important to note that the technically simple approach of streptomycin resistance transformation appears to reflect general gen omic differences and similarities

Although the results of pulse RNA DNA hybridization are taxonomically inconclusive by themselves because of the limited number of strains and combinations of RNA and DNA donors used, they are of value when con sidered together with the other studies which also showed that the strains employed here are genetically representative of their species designations

It is established that the classical *Moraxella* and *Neisseria* concepts as they are presented in Bergey s Manual (2) are related and partially show much higher intergroup than intragroup nucleic acid homologies It ap pears therefore very reasonable to transfer genus *Moraxella* from its present family Bru cellaceae (2) to Neisseriaceae as proposed by *Henriksen & Bøvre* (19)

Intragenus relationship between *N catar rhalis N caviae* and *N ovis* is commonly ac cepted The two latter may have relatively high affinities even for species of the same genus as shown in cultural and biochemical studies genetic transformation and by base determination (1 6 7) The three species are called false neisseriae because of their low affinity to other oxidase positive cocci (true neisseriae) in genetic transformation (6 13) and a distinctly lower G + C content of their DNAs (7 13) The hybridization results of this and other investigations (23 24) support this distinction The overlapping compatibi lities in pulse RNA DNA hybridization ge netic transformation (6) and in terms of DNA base composition (7) between false neisseriae and rodshaped moraxellae show that they all could belong to one and the same genus *i e* genus *Moraxella* (19) The hybridization results further support the in clusion of the rodshaped strain M2 in the revised true genus *Neisseria* (19) as *N elongata* (10) This genus would thereby also contain both coccal and rodshaped organisms

A question of great taxonomic importance is whether the genus *Moraxella* and the genus *Neisseria* when defined on the basis of ma cromolecular affinities (10 19) can be said to reveal some indication of mutual relation ship favouring their position as members of one family *i e* Neisseriaceae Such affinities were not found in the transformation studies and it is not known whether the slight inter actions observed in the present investigation may indicate family relationship However there seem to be some slight DNA homologies between the false and true neisseriae which were disclosed in DNA DNA hybridi zation (23) For the time being it is therefore not advisable to separate the two groups in different families

The present limited study does not provide sufficient data for discussion of the special taxonomic problem concerning the deviating *M kingii* species (18 19) nor the relations between oxidase positive and negative or ganisms

The main part of the work was performed during a *U S Public Health Service International Post doctoral Research Fellowship* Special thanks are due to Dr *W S ybalski* for invaluable technical advice and comments and for providing good re search facilities at McArdle Laboratory University of Wisconsin

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cially when compared with some recent DNA DNA hybridization studies (17 20 21, 23 29) The occasional occurrence of measurements of the same relations by both methods indicate however that the techniques of DNA DNA hybridization reveal higher values of homology at least under some conditions For instance *Kingsbury* (23) found that *Neisseria catarrhalis* DNA is 28 per cent homologous with *N flavescens* DNA, as compared with 0 6 per cent in the precent study However, increased incubation temperature resulted in a marked decrease of some other interspecies DNA DNA hybrids tested (24) so that the figures compared are centainly not representative of the difference between the two modes of hybridization

In DNA DNA hybridization the labelled DNA fragments are often longer than the homologous regions and the unmatched nucleotide sequences cannot at present be selectively eliminated from the hybrid after annealing The latter obstacle can be avoided in RNA DNA hybridization by means of RNase However some taxonomic RNA DNA hybridization studies have not included RNase treatment of the hybrids before assay As in DNA DNA hybridization the controls of specificity most often are 1) the relatively low affinity of distantly related DNAs or 2) little binding of the radioactive component in the absence of denatured unlabelled DNA Unfortunately such controls do not tell much about the base to base specificity of more active heterologous reactions in the system It has been shown in bacteriophages and bacteria that even limited nucleic acid homology of two organisms may be enough to raise a heterologous RNA DNA hybridization up to a considerable level when RNase treatment is omitted whereas a DNA free control or an autologous reaction may be almost unaffected by the presence or absence of RNase (11 30)

Affinities even as low as in th 2–6 per cent range seem to be compatible th intra generic relationships in a pulse I A DNA or mRNA DNA hybridization s m with controlled methodology including the use of RNase Evidence in favour of this has been revealed in genus *Bacillus* (15) Also only 5–11 per cent homology was found in a careful mRNA DNA hybridization study of the phages λ and φ80, which must be considered related both from genetic recombination data and from the molecular organization and function of their genomes (25) Although there exists no proof for its superiority over simple pancreatic RNase treatment the combined use in the present study of pancreatic RNase (which attacks nucleotide sequences next to pyrimidine nucleotides) and T_1RNase (attacking sequences only adjacent to guanylic acid) should ensure that only minimal amounts of noncomplementary structures are being measured as hybrids

Because directly comparable reports are scarce the actual percentages of heterologous out of autologous hybridization are of less interest than the relative magnitudes of these ratios as discussed below

With due attention to the fact that most observations were made with one strain only from each species the present hybridization results can be summarized as follows 1) The oxidase positive rodshaped *Moraxella nonliquefaciens M liquefaciens M lacunata* and *M bovis* (unequivocal moraxellae) have as high or higher affinity to false neisseriae (*N catarrhalis N caviae N ovis*) as have the latter among themselves 2) *M nonliquefaciens* and false neisseriae' show little reactivity with *N cinerea N flavescens* and *N flava* (representatives of true neisseriae) 3) *N elongata* a rodshaped organism has also low affinity to *M nonliquefaciens* whereas the compatibility with *N flava* is high 4) *M liquefaciens M lacunata* and *M bovis* have particularly high affinities to *M nonliquefaciens* 5) The compatibility of *M bovis* with *N ovis* is almost as high as with *M nonliquefaciens* 6) *M osloensis* and *M phenylpyruvica* have lower affinity to *M nonliquefaciens* but still more than have *Acinetobacter lwoffi M kingii N cinerea N flavescens N flava* and *N elongata*

Generally there is consistency between the results of hybridization and genetic transfor

mation (Tables 1 and 2) in almost the same combinations of strains (5 6, 8 9 10 18) The correspondence between genetic trans formation data and DNA base ratios has been discussed previously (5 7, 10) It is parti cularly important to note that the technically simple approach of streptomycin resistance transformation appears to reflect general gen omic differences and similarities

Although the results of pulse RNA DNA hybridization are taxonomically inconclusive by themselves because of the limited number of strains and combinations of RNA and DNA donors used, they are of value when con sidered together with the other studies which also showed that the strains employed here are genetically representative of their species designations

It is established that the classical *Moraxella* and *Neisseria* concepts as they are presented in Bergey s Manual (2) are related and partially show much higher intergroup than intragroup nucleic acid homologies It ap pears therefore very reasonable to transfer genus *Moraxella* from its present family Bru cellaceae (2) to Neisseriaceae as proposed by *Henriksen* & *Bøvre* (19)

Intragenus relationship between *N catar rhalis N caviae* and *N ovis* is commonly ac cepted The two latter may have relatively high affinities even for species of the same genus as shown in cultural and biochemical studies genetic transformation and by base determination (1 6 7) The three species are called false neisseriae because of their low affinity to other oxidase positive cocci (true neisseriae) in genetic transformation (6 13) and a distinctly lower G + C content of their DNAs (7 13) The hybridization results of this and other investigations (23 24) support this distinction The overlapping compatibi lities in pulse RNA DNA hybridization ge netic transformation (6) and in terms of DNA base composition (7) between false neisseriae and rodshaped moraxellae show that they all could belong to one and the same genus *i e* genus *Moraxella* (19) The hybridization results further support the in clusion of the rodshaped strain M2 in the revised true genus *Neisseria* (19) as *N elongata* (10) This genus would thereby also contain both coccal and rodshaped organisms

A question of great taxonomic importance is whether the genus *Moraxella* and the genus *Neisseria* when defined on the basis of ma cromolecular affinities (10 19) can be said to reveal some indication of mutual relation ship favouring their position as members of one family *i e* Neisseriaceae Such affinities were not found in the transformation studies and it is not known whether the slight inter actions observed in the present investigation may indicate family relationship However there seem to be some slight DNA homologies between the false and true neisseriae which were disclosed in DNA DNA hybridi zation (23) For the time being it is therefore not advisable to separate the two groups in different families

The present limited study does not provide sufficient data for discussion of the special taxonomic problem concerning the deviating *M kingii* species (18 19) nor the relations between oxidase positive and negative or ganisms

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characteristic result of the enzyme activity After developing the zones of proteinase activity appear as distinct white spots in the otherwise transparent agar medium From one to three proteolytic fractions have been demonstrated in the different materials investigated (Fig 1 and Fig 2) On the other hand, one single spot cannot with certainty be considered to represent only one proteinase until electrophoretical and possibly, serological exclusion of interfering enzyme fractions has been carried out at varying pH values Such investigations are however not practicable in the screening of a large number of bacterial proteinases

The electrophoretical patterns support the theory advanced by *Dahle* (1969 b) that *Ae liquefaciens* is able to produce two proteinases (A and B) while *Ae salmonicida* only has the capacity to produce one proteinase (B) under the conditions examined (Figs 1a and 1b) Furthermore the identical migration rates of the B proteinases produced by the two organisms are in support of the finding that the two enzymes are identical or closely related For diagnostic purposes the zymogram technique can be used to differentiate between the two aeromonads

The sensitivity of the method is of importance when the material contains one proteinase which is quantitatively in excess in relation to others (Fig 2c) It is not feasible however to set any lower limit for the amount of enzyme to be detected in this way but as regards bovine trypsin the results indicate that minimal amount is 0 05 µg as compared with the 100 µg involved in the method described by *Uriel* (1960) The difference in sensitivity can be due to the different principles of the developing systems Thus the CP reaction seems to be preferable to methods based on a primary reaction of the enzymes with the substrate followed by a secondary staining procedure

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RABBIT ANTI RAT LYMPHOCYTE SERUM CHARACTERIZATION OF THE *IN VITRO* LYMPHOCYTOTOXIC ANTIBODY WITH REFERENCE TO THE IMMUNIZATION PROCEDURE AND THE ANTIGEN DOSE

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The immunoglobulin classes of the *in vitro* active lymphocytotoxic antibodies of different antilymphocyte sera have been investigated Rabbits were immunized intravenously with different numbers of rat spleen cells biweekly for 1–4 weeks The effect of the antigen dose and the length of the immunization course was investigated Lymphocytotoxic anti bodies were found both in the IgG and in the IgM fraction of the antisera The relative contribution of the cytotoxic activity of the IgG and the IgM fraction towards the final cytotoxic activity of the crude antiserum depended on the antigen dose and the length of the immunization course Rabbits immunized with the antigen mixed with Freund s adjuvant intramuscularly and given a booster dose intravenously showed the same pattern as rabbits immunized intravenously

Heterologous anti lymphocyte serum (ALS) is a strong immunosuppressive agent (for review see 12) The immunosuppressive activity of a rabbit anti mouse lymphocyte serum as tested by its ability to delay the rejection of skin allografts has been shown to reside only in the IgG fraction of the anti serum (13) Others have confirmed that the IgG fraction of different antisera contains immunosuppressive activity (2 10 15 18)

Ability of ALS to act cytotoxically towards target cells *in vitro* was first shown by *Metchnikoff* (16) and its *in vitro* agglutinating ability by *Pappenheimer* (17) Numerous workers have confirmed that crude ALS contains lymphocytotoxic and lymphoagglutinating antibodies (1 5 8) Crude ALS also stimulates blastoid transformation of cultured lymphocytes (22) inhibits rosette formation (4) and opzonizes lymphocytes (10)

The results of these different *in vitro* tests do not always correlate with the ability of the particular ALS to prevent rejection of skin allografts Some workers have claimed that the results of the rosette inhibition test and the opzonizing test correlate with immunosuppressive activity *in vivo* (4

No definite correlation

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TABLE 3 *Influence of Increasing Antigen Dose on the Cytotoxic Titre*

Antigen dose	Rabbit no	Serum	2 ME treatment	IgM fraction	IgG fraction
1×10^6	80	64	16	<4	<4
	81	128	32	<4	<4
1×10	65	512	64	16	8
	79	1024	64	8	8
1×10^8	63	1024	128		
	67	1024	256	16	16

In vitro lymphocytotoxic activity of different antilymphocytesera raised with increasing antigen dose by intravenous immunization biweekly for 4 weeks Comparison of activity of serum 2 mercaptoethanol treated serum and of IgM and IgG fractions

antisera All sera showed some reduction of their lymphocytotoxic strength Sera from the rabbits immunized with 1×10^7 spleen cells were reduced from a fairly high lympho cytotoxic titre of 512 or 1024 to 64, showing that a considerable amount of the lympho cytotoxic activity of these sera resided among the mercapto sensitive antibodies These re sults correlated with the separation on column chromatography

3 *Influence of Freund s Adjuvant on Immu ni ations with different Antigen Doses*

The lymphocytotoxic titre of antisera raised with Freund s adjuvant showed little differ ence between sera raised with 1×10^7 and 1×10^8 spleen cells (Table 4) The sera raised with 1×10^8 spleen cells contained mercapto resistant lymphocytotoxically active antibody than did those raised with 1×10 cells One serum showed no dimi nution in cytotoxic activity after reduction and alkylation

4 *Influence of Increasing Number of Immuni ations with Standard Antigen Dose*

Considerable variation in lymphocytotoxic titre after standard immunization was found within each group of animals (Table 5) Relatively high titres were found after 4 immunizations employing as few cells as 1×10^7 This serum and most of the other sera resulting from either 4 or 6 immuniza tions of 1×10^7 number of spleen cells con tained however cytotoxic activity that was destroyed by treatment with mercaptoet hanol and subsequent alkylation The general tendency that sera raised employing 1×10^7

TABLE 4 *Influence of Freund s Adjuvant on the Cytotoxic Titre*

Antigen dose	Freund s adjuvant	Rabbit no	Serum	2 ME treated serum
1×10^7	+	82	128	32
	+	83	256	32
	—	65	1024	64
	—	79	512	64
1×10^8	+	77	128	64
	+	78	128	128
	—	63	1024	128
	—	67	1024	256

Comparison of *in vitro* lymphocytotoxic activity of antilymphocyte sera raised with and without Freund s adjuvant before and after treatment with 2 mercaptoethanol

TABLE 5 *Influence of Increasing Number of Immunizations on the Cytotoxic Titre*

No of weeks	Antigen dose	Rabbit no	Serum	2 ME treated serum	IgM fraction	IgG fraction
1	1×10	90	64	16	8	4
		93	32	8		
2	1×10^7	91	512	32	16	4
		96	64	16		
		98	128	16		
3	1×10^7	92	512	128	8	32
		95	256	64	4	8
		97	256	32	4	4

In vitro lymphocytotoxic activity of different antilymyphocytsera raised with standard antigen dose and increasing number of immunizations Comparison of activity of serum 2 mercaptoethanol treated serum and of IgM and IgG fractions

spleen cells contained mainly mercapto sensitive antibody activity was confirmed when testing the fractions of antisera after fractionation on Sephadex G 200

DISCUSSION

The cytotoxic test used in the present study gave very consistent titres of the antisera tested The addition of 0 2 per cent Trypan Blue to the pellet resulted in very consistent colouring of the cells The concentration of Trypan Blue corresponds with that suggested by *Engelfriet & Britten* (7) Different lower limits of per cent stained cells have been used to estimate the titre of an antiserum (1 14) The sharp fall of the cytotoxicity curve over 1–2 double dilutions meant that the choice of 10 25 or even 50 per cent stained cells as the lower limit did not greatly influence the titre

Reduction of IgM antibodies with 2—mercaptoethanol results in loss of all antibody activity while the antigen binding capacity of IgG antibodies is not destroyed This difference has been used to distinguish these two classes of antibodies (11) In this study it was found that reduction with equal volumes of 0 4 M 2—mercaptoethanol resulted in total abolition of antibody activity of the antisera while reduction carried out with equal volumes of 0 2 M 2—mercaptoethanol resulted in the retention of some antibody activity The picture of the antisera containing antibody activity both of mercaptoethanol sensitive and mercaptoethanol resistant nature as was obtained employing a final molarity of 0 1 M 2—mercaptoethanol correlated with the results of the column chromatography Reduction with a final molarity of 0 2 M may have resulted in breaking of disulphide bonds of the IgG molecule with concomitant loss of antibody activity

On the other hand however mercaptoethanol treated antiserum was shown to be unable to haemolyse rat red blood cells in the presence of complement Agglutination occured instead Furthermore both the IgM and the IgG fraction lost their cytotoxic activity on reduction with mercaptoethanol while haemagglutinating ability was retained in the IgG fraction Mercaptoethanol treatment thus seems to have interfered with the ability of the different antibodies to bind complement This is in correspondence with the finding that mercaptoethanol treatment resulted in loss of ability of human 7 S gamma globulin to fix complement (20) Mercaptoethanol treatment of an antiserum might thus give false indications as to the presence and content of antibodies belonging to the IgG class if the method for estimation of the antibodies involves complement In the present study most of the antisera lost some cytotoxic strength on reduction with a final molarity of 0 1 M 2—mercaptoethanol *Ia*

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GEL PRECIPITATION REACTIONS BETWEEN MEASLES ANTIGENS AND SERA OF PATIENTS WITH MULTIPLE SCLEROSIS

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The gel precipitation reactions between measles antigens and serum specimens of 49 patients with multiple sclerosis and 49 age matched controls were studied At least one gel precipitation line was obtained in 76 per cent of the control sera and in 94 per cent of the patient sera ($p < 0.01$) Five patient sera produced four or five strong precipitation lines against measles antigens but no lines against the noninfected control antigens In the serum specimens of the control group not more than three faint precipitation lines against measles antigens were detected Comparison of the gel precipitation reactions between the sera of these five patients with multiple sclerosis and of a patient with subacute sclerosing panencephalitis indicated an almost identical gel precipitation pattern

Several diseases associated with chronic demyelination may have a virus aetiology Both indirect and recently obtained direct evidence indicate that subacute sclerosing panencephalitis (SSPE) is caused by measles virus infec This view is supported by histological findings (7) serological tests (4) and isolation of measles virus from brain biopsies (11)

Multiple sclerosis another demyelinating disease with onset later than that of SSPE mostly in the third and fourth decades, may also have a viral aetiology (12) Since 1962 several reports have indicated increased levels of measles antibodies in patients with multiple sclerosis (1 3 10) In addition electron microscopy studies on brain biopsies of patients with multiple sclerosis have revealed changes similar to those found in SSPE (5 13)

Recent demonstration in this laboratory of strong gel precipitation (GP) reactions between serum specimens of SSPE patients and measles antigen (9) prompted an attempt to demonstrate and characterize measles antibody in sera of patients with multiple sclerosis The present report describes the precipitation reactions between measles antigen and serum specimens of patients with multiple sclerosis and matched controls These reactions are compared with those of an SSPE patient

MATERIAL AND METHODS

Serum specimens The serum specimens of 49 patients with multiple sclerosis and 49 controls matched [illegible] birth date and place of residence were ra[illegible]lected from a *larger series* collected [illegible]tailed data relating to the

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material have been published elsewhere (10) The serum specimen of an SSPE patient was collected from a 14 year old boy with typical clinical and serological findings including SSPE complexes in EEG high gamma globulin level in CSF and increased levels of measles antibody titres in serum (CF ≧ 256) and in CSF (CF = 32)

Gel precipitation antigens Roux bottles containing LLC MK2 cells (monkey kidney cell line) and U-cells (human amnion cell line) were maintained with BME (diploid) medium prepared from GIBCO (New York) powder medium and supplemented with 5 per cent horse serum (cell pack antigens) or 0 4 per cent bovine albumin fraction V Armour Pharmaceutical Co Ill (total harvest antigen) After inoculation with undiluted measles virus (6) the cultures were incubated at 37 C until complete degeneration of cell had occurred 5 to 10 days after virus inoculation

For preparation of the cell pack antigens cells were mechanically detached from the glass surface centrifuged at low speed and washed twice in phosphate buffered saline pH 7 4 (PBS) A 30 per cent suspension of the washed cells was made in PBS and sonicated in a Raytheon sonic oscillator model DF 101 until at least 90 per cent of the cells were disrupted according to visual examination under a phase contrast microscope The sonicated material was clarified by low speed centrifugation for 15 minutes The supernatant containing the gel precipitation antigen was stored at −20 C The control antigens were prepared in the same way except that no virus was inoculated The antigens were resonicated for 2 minutes immediately before use

For the total harvest antigens the cells were mechanically detached and the whole cultures sonicated The cell debris was removed by low speed centrifugation and the supernatant concentrated by forced dialysis against polyethylenglygol 20000 The bovine albumin was separated from the concentrated preparations by gel filtration through Sephadex G 200 column The fractions without albumin were concentrated by forced dialysis against polyethylenglycol The concentrated preparations were sonicated for 5 minutes and stored and resonicated in the same way as the cell pack antigen

Gel precipitation test Gel precipitation tests were carried out on 5 cm × 5 cm glass photographic slides with a microtechnique reported in detail elsewhere (14) The volume of reactant used was 25 μl, and 1 per cent agarose was used for diffusion

Haemagglutination inhibition test The preparation of the antigens and the microtechnique used in the HI test have been reported elsewhere (10)

RESULTS

The number of precipitation lines obtained using a total harvest antigen prepared from measles infected LLC MK2 cells against the sera tested is shown in Table 1 At least one gel precipitation line was obtained in 76 per cent of the control sera and in 94 per cent of the patient sera the difference being statistically significant ($p < 0.01$) in Student's t test

The highest number of gel precipitation lines in the controls was 3 whereas 4 or 5 lines were obtained with 5/49 (12 per cent) in sera of patients with multiple sclerosis The gel precipitation lines of the serum specimens of the controls were usually weak but in the sera of the 5 patients with multiple lines 2 or 3 of the lines were considerably stronger as seen in Fig 1 These 5 specimens were also tested with a control antigen of LLC MK2 cells (cell pack antigen) and no precipitation line was detected

The number of lines in the gel precipitation test was not clearly correlated with the measles haemagglutination inhibition titres in sera of patients or controls (Fig 2) One of the multiple sclerosis specimens with 5 precipitation lines and one of the control specimens with 2 lines and the serum of the SSPE patient were tested against measles

TABLE 1 *Gel Precipitation Reactions of Sera From Patients with Multiple Sclerosis and from the Controls against a Total Harvest Antigen Prepared from Measles Infected LLC MK2 Cells*

Group of sera	Number of GP line						Per cent positive
	0	1	2	3	4	5	
Control group	12	23	9	5	0	0	37/49 = 75 %
MS group	3	33	5	3	2	3	46/49 = 94 %

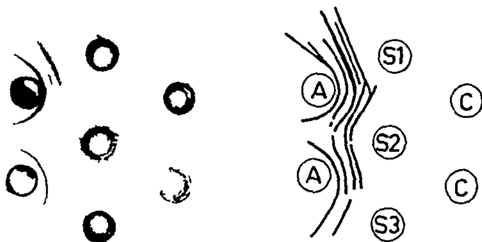


Fig 1 Gel precipitation reactions of measles and control antigens against serum specimens from patients with multiple sclerosis subacute sclerosing panencephalitis and a serum specimen from the control group A = measles antigen C = control antigen S1 = serum from a patient with MS S2 = serum from a patient with SSPE S3 = serum from the control group

antigen (total harvest antigen) and a control antigen on the same precipitation slide (Fig 1) The typical pattern of at least 5 precipitation lines is seen between the SSPE serum and measles antigen and three of the five lines produced by the multiple sclerosis serum coalesce completely and two partially with the SSPE lines Two faint lines produced by the control serum coalesce with two of the lines produced by serum of the patient h multiple sclerosis No lines were produced by the control antigen against any of the three serum specimens

The SSPE serum was also tested against cell pack antigens prepared from measles infected U cells and LLC MK2 cells These two cell pack antigens yielded a precipitation pattern similar to that obtained with the total harvest antigen prepared in LLC MK2 cells Control antigens prepared from U cells and LLC MK2 cells produced no recipitation line against the SSPE serum

DISCUSSION

...ts of the present study inc ate that ...ts with multipl sclerosis had measles specific gel precipitating antibodies almost identical with those in SSPE patients Since similar strong gel precipitation reactions were not found in the normal population and have not been found earlier in convalescent specimens of measles patients the results obtained provide additional evidence of an association between measles virus infection and multiple sclerosis in some of the patients

The nature of the precipitation lines of SSPE serum against measles antigens has not been clarified but the established measles aetiology of SSPE (11) strongly suggests that the multiple lines are caused by measles antibodies produced by persistent measles virus infection in the brain cells of the patients The number of lines in the gel precipitation test indicate in general, either the number of antigenic determinants immunogenic to the patient or the aggregation of small size antigens in the preparations The antigenic determinants in the gel precipitation antigens used in the present study represent proteins of virus particles or virus specific nonstructural proteins developed in the cell cultures during virus infection The techni

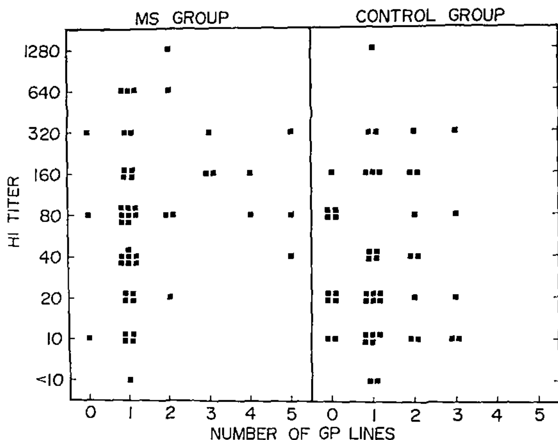


Fig 2 Relationship between the HI titres and the number of GP lines in serum specimens from patients with multiple sclerosis and from the cont ol group

que by which the antigens were prepared ensured the presence of nonstructural proteins in the gel precipitation antigens Evidence of the virus and not cell specificity of these gel precipitation antigens is afforded by the similar GP reactions in SSPE serum with measles antigens prepared in cells of human and monkey origin It will be interesting to ascertain the type of precipitation reactions produced by sera of patients with multiple sclerosis against concentrated purified and disintegrated virus particles

Long term follow up of the occurrence of gel precipitating measles antibodies in patients with SSPE multiple sclerosis measles encephalitis and uncomplicated measles is required It has been demonstrated that a peak incidence of rubella gel precipitation antibodies occurs 3 to 4 months after the illness (14) Animal models with herpes virus hominis (8) and respiratory syncytial virus (2) indicate late development of gel precipitation antibodies and a short period with peak incidence of precipitation lines

Of the multiple sclerosis sera tested only about one tenth gave gel precipitation reactions different from those of normal sera Evidence is as yet too limited to decide whether this is an indication of multiple aetiology of the disease or of an unknown time factor governing the occurrence of the peak incidence of gel precipitating antibodies and possibly correlated with the remitting and relapsing course of this disease The 5 sera of patients with multiple precipitation lines represented neither a particularly active phase of the disease nor a speci group among the patients stud

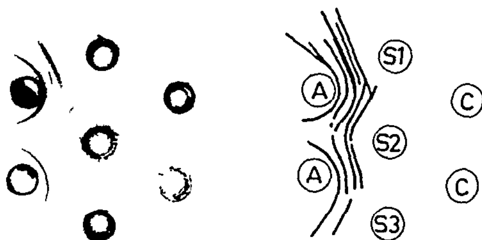


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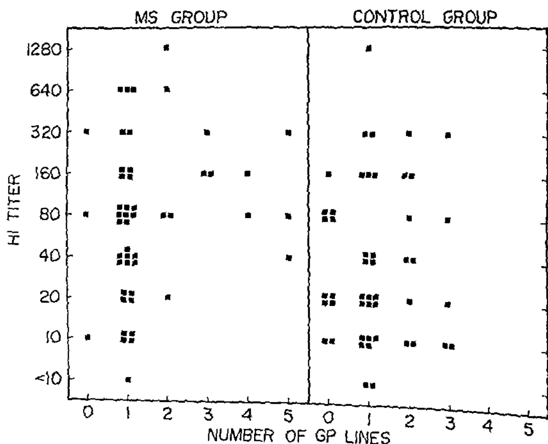


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rabbits were immunized with saline suspension from overnight (37 C) broth agar plates density about 10^9 organisms per ml formaldehyde was added to a final concentration of 0 05 per cent Injection doses were 0 2 0 5 1 0 1 5 and 2 0 ml intravenously at 4–5 day intervals Bleeding was carried out 7 or 8 days after the final injection The sera from the individual rabbits were kept separate and preserved by the addition of merthiolate

Absorption

The traditional procedure for the production of pure L from OK antisera is as follows After overnight incubation at 37 C the culture from 14 cm diameter broth agar plates is suspended into saline 10 plates or 50 ml saline The suspension is heated at 100 C for 2 hours divided into two and centrifuged at 8000 g for 30 minutes Generally the culture is washed once One pellet is mixed with 10 ml anti OK serum diluted 1 10 The mixture is kept overnight at 4 C On centrifugation the supernatant is mixed with the other pellet and left overnight at 4 C After centrifugation the supernatant constitutes the absorbed anti L serum. Undiluted or L antiserum concentrated to 50 per cent of original volume was necessary for immunoprecipitation and was obtained in the following way 4 ml undiluted or concentrated OK antiserum was absorbed with culture from ten 14 cm diameter plates on four successive occasions (when the concentrated serum is mentioned in the following this refers to the serum which was concentrated to 50 per cent of the original volume)

Agglutination technique

Bacterial agglutinations were carried out in small tubes (10 x 70 mm) Equal volumes (0 2 ml) of antiserum diluted two-fold in saline and antigens were mixed The antigens were saline suspensions of bacteria from broth agar plates incubated overnight at 37 C density about 10^9 organisms per ml This suspension was used either after the addition of formaldehyde (K agglutinations) or after being heated to 100 C for 1 hour (O agglutinations) Titrations for K agglutinations were kept at 37 C for 2 hours and read after standing overnight at room temperature while O agglutinations were incubated at 50 C for ?0 hours in waterbath

Antigenic preparations

For immuno-diffusion growth from 1 cm diameter plates was suspended in saline 1 n er plate (between 10^{10} and 10^{11} organisms per l) and cated in waterbath at 60 C for 20 utes or 00 C for 1 hour For passive haemag tination 10 ml saline as sed per plate and l ing was carried out for 2 hours In both cases the cells were removed from the extracts by centrifugation at 27000 g for 15 or 30 minutes

Immuno Diffusion Methods

Double diffusion in gel was carried out by two modified Ouchterlony techniques one as described by *Hanson* (2) and the other a paper disc method described previously (2?) One per cent (Behring) Reinagar in 0 066 M phosphate buffer (pH 7) containing 0 065 per cent azide and 0 11 per cent ethylenediaminetetra acetic acid (EDTA) was poured into Petri dishes (14 cm diameter) to give a layer about 1 mm thick Filter paper discs (6 mm) were soaked with sera or extracts The discs were placed on the agar in an appropriate pattern The closed Petri disches were stored in plastic bags to reduce evaporation and were incubated for one day at 37 C and for 4–10 days at room temperature The development of precipitin lines was inspected daily

The immuno-electrophoretic technique was that of *Scheidegger* (20) using the apparatus commercially manufactured by LKB of Sweden Three glass slides were coated with 9 ml 1 5 per cent agar Noble in veronal buffer pH 8 6 ionic strength 0 05 μ Wells and troughs were cut with the LKB device The agar from the troughs was sucked off and the troughs filled with 1–1 5 μl antigen solution Veronal buffer pH 8 6 ionic strength 0 05 was used in the electrophoretic chamber The electrophoretic separation was performed at a voltage of 7 V/cm for 2 hours The agar in the well was lifted out and the well filled with about 150 μl undiluted (or concentrated) antiserum The slides were left in a moist chamber overnight The results were recorded then the slides were washed for 2 days in saline and on the third day washed in distilled water before staining with Amido schwarz. All results of immuno-diffusion tests are based on replicate experiments using analogous antisera from different rabbits prepared at the same or at different times

Passive Haemagglutination Technique (HA)

Sheep red blood cells were treated with formaldehyde according to the method described by *Weinbach* (?4) Bacterial antigens were adsorbed on to these with or without preceding treatment wiht tannic acid The method employed for the test without tannic acid treatment (HA) was based on the report of *Neter et al* (15) 1 ml packed cells was incubated together with 49 ml antigenic extract undiluted or diluted 1 10 or 1 100 in a 37 C waterbath The results given in the tables are exclusively those carried out with *undiluted extracts* After 30 minutes the red blood cells were washed

three times in buffered saline (0.01 M phosphate buffer). The sensitized cells were kept as a 2 per cent suspension in buffered saline. 0.2 ml of this suspension was added to the same amount of two-fold serial dilutions of the antisera in perspex trays. The reactions were recorded after incubation for 3 hours at 37 C. The controls were coated cells in normal rabbit serum (NRS).

The test carried out with tannic acid treated red cells (HAt) was performed by a modification of the method described by *Boyden* (1). 2 ml formalinized packed red sheep cells were washed once and suspended in 40 ml buffered saline. 4 ml freshly prepared tannic acid solution (1:1000) and 56 ml buffered saline were added rapidly and the mixture incubated in a waterbath at 37 C (shaken occasionally). After 15 minutes the mixture was centrifuged and washed once in 100 ml buffered saline. The tannic acid treated cells (2 ml) were resuspended in 50 ml antigenic extract and left in water bath at 37 C for 30 minutes, then centrifuged and washed once in 100 ml buffered saline (+ 1.5 per cent NRS absorbed with sheep red cells). The cells were kept as a 10 per cent suspension in buffered saline (+ 1.5 per cent absorbed NRS) but used in 2 per cent suspension, 0.2 ml being mixed with 0.2 ml antiserum. The HAt reaction was read after incubation overnight in the refrigerator. *All* centrifugations in connection with both tests were carried out for 10 minutes at 480 g. When the HA and the HAt titrations were compared directly, the procedure for the HAt was followed, half of the red cells being treated with tannic acid for the HAt test and the other half with buffered saline for the HA test.

RESULTS

Bacterial Agglutination Technique (BA)

Demonstration of L antibodies in OK serum absorbed undiluted and diluted 1:10 with homologous boiled culture. OK antisera of three K antigen test strains O4:K12(L):H–, O1:K51(L):H– and O4:K52(L):H–, were absorbed by the traditional method us

TABLE 1. *Comparison of Bacterial Agglutination (BA) and Passive Haemagglutination (HA) in OK Antisera Absorbed with Homologous Boiled Cultures*

Test		Antigen	OK antiserum K12 Unabsorbed	OK antiserum K12 Absorbed undiluted	OK antiserum K12 Absorbed 1:10	O antiserum K12
BA	K12	unheated	2560	1024	1280	80
		100 C	1280	< 2	< 20	≥ 5120
HA	K12	60 C extr *	640	2	20	< 20
		100 C	1280	< 2	< 20	640
			OK antiserum K51 Unabsorbed	Absorbed undiluted	Absorbed 1:10	O antiserum K51
BA	K51	unheated	640	4	40	20
		100 C	≥ 5120	< 2	< 20	≥ 5120
HA	K51	60 C extr	1280	2	20	< 20
		100 C	2560	< 2	20	640
			OK antiserum K52 Unabsorbed	Absorbed undiluted	Absorbed 1:10	O antiserum K52
BA	K52	unheated	320	8	40	< 20
		100 C	≥ 5120	< 8	< 20	≥ 5120
HA	K52	60 C extr	320	2	20	< 20
		100 C	≥ 10240	< 4	< 20	5120

* Extracts used for sensitization of erythrocytes, see Methods. Titre is reciprocal value of the highest serum dilution showing clumping visible to the naked eye.

rabbits were immunized with saline suspension from overnight (37 C) broth agar plates density about 10^9 organisms per ml formaldehyde was added to a final concentration of 0 05 per cent Injection doses were 0 2 0 5 1 0 1 5 and 2 0 ml intravenously at 4–5 day intervals Bleeding was carried out 7 or 8 days after the final injection The sera from the individual rabbits were kept separate and preserved by the addition of merthiolate

Absorption

The traditional procedure for the production of pure L from OK antisera is as follows After overnight incubation at 37 C the culture from 14 cm diameter broth agar plates is suspended into saline 10 plates in 50 ml saline The suspension is heated at 100 C for 2 hours divided into two and centrifuged at 8000 g for 30 minutes Generally the culture is washed once One pellet is mixed with 10 ml anti OK serum diluted 1 10 The mixture is kept overnight at 4 C On centrifugation the supernatant is mixed with the other pellet and left overnight at 4 C After centrifugation the supernatant constitutes the absorbed anti L serum Undiluted or L antiserum concentrated to 50 per cent of original volume was necessary for immunoprecipitation and was obtained in the following way 4 ml undiluted or concentrated OK antiserum was absorbed with culture from ten 14 cm diameter plates on four successive occasions (when the concentrated serum is mentioned in the following this refers to the serum which was concentrated to 50 per cent of the original volume)

Agglutination technique

Bacterial agglutinations were carried out in small tubes (10 x 70 mm) Equal volumes (0 2 ml) of antiserum diluted two fold in saline and antigens were mixed The antigens were saline suspensions of bacteria from broth agar plates incubated overnight at 37 C density about 10^9 organisms per ml This suspension was used either after the addition of formaldehyde (K agglutinations) or after being heated to 100 C for 1 hour (O agglutinations) Titrations for K agglutinations were kept at 37 C for 2 hours and read after standing overnight at room temperature while O agglutinations were incubated at 50 C for 20 hours in waterbath

Antigenic preparations

For immuno-diffusion growth from [illegible] m diameter plates was suspended in saline 1 m[illegible]er plate (between 10^{10} and 10^{11} organisms pe[illegible]l) and heated in waterbath at 60 C for 20 [illegible]utes or 100 C for 1 hour For passive haemag[illegible]unation 10 ml saline was [illegible]ed per plate and [illegible]ing was carried out for 2 hours In both cases the cells were removed from the extracts by centrifugation at 27000 g for 15 or 30 minutes

Immuno Diffusion Methods

Double diffusion in gel was carried out by two modified Ouchterlony techniques one as described by *Hanson* (2) and the other a paper disc method described previously (22) One per cent (Behring) Reinagar in 0 066 M phosphate buffer (pH 7) containing 0 065 per cent azide and 0 11 per cent ethylenediaminetetra acetic acid (EDTA) was poured into Petri dishes (14 cm diameter) to give a layer about 1 mm thick Filter paper discs (6 mm) were soaked with sera or extracts The discs were placed on the agar in an appropriate pattern The closed Petri disches were stored in plastic bags to reduce evaporation and were incubated for one day at 37 C and for 4–10 days at room temperature The development of precipitin lines was inspected daily

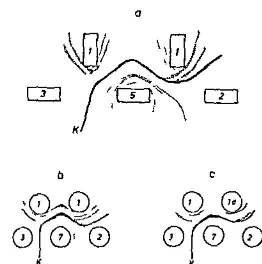
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Passive Haemagglutination Technique (HA)

Sheep red blood cells were treated with formaldehyde according to the method described by *Weinbach* (24) Bacterial antigens were adsorbed on to these with or without preceding treatment wiht tannic acid The method employed for the test without tannic acid treatment (HA) was based on the report of *Neter et al* (15) 1 ml packed cells was incubated together with 49 ml antigenic extract undiluted or diluted 1 10 or 1 100 in a 37 C waterbath The results given in the tables are exclusively those carried out with undiluted extracts After 30 minutes the red blood cells were washed

K antigen has a high electrophoretic mobility towards the anode The 60° and 100°C extracts show a slight difference in the mobility of this antigen perhaps indicating some inhomogeneity of the K antigen extracted at 100°C Heating of the 60°C extract to 100°C did not cause this change in the mobility of the K antigen

The O antigen precipitation lines were not as distinct as the K lines They appeared as one two or more bands particularly between the basins or discs containing unabsorbed OK antiserum and OK antiserum absorbed by the



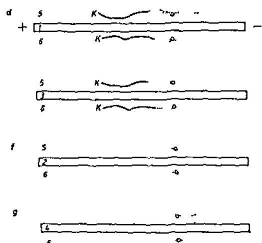


Fig 1 a = double diffusion in gel using wells (2) b and c = double diffusion in gel using paper discs (22) d e f and g = immuno-electrophoretic analysis 1 or 1a = OK antiserum K12 2 or 2a = OK antiserum K12 absorbed with homologous 100 C culture 3 or 3a = OK antiserum K12 absorbed with heterologous 100 C culture possessing the same O antigen as K12 4 = O antiserum of K12 5 = 60 C extract of K12 6 = 100 C extract of K12 7 = disc soaked with surface plate culture (60 C) 1 and 1a etc = comparison of antisera from different rabbits * = 100 C extract of K5?

Fig 2 See Fig 1 except that K12 should be read K51

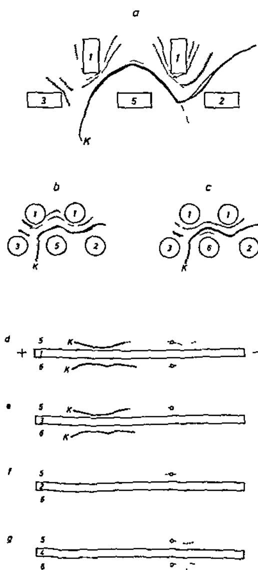


TABLE 5 *Passive Haemagglutination Test (HAt) Using Tannic Acid Treated Erythrocytes Comparison with the HA Test Sensitization with 60 C Extract of K12*

Antigen (60 C extract used for sensitization)	OK antiserum K12		O anti serum K12	NRS*
	unabsorbed	abs undiluted by homol 100 C washed culture		
Tannic acid treated cells	≥ 655 360	20 480	160	80
Non treated cells	2 560	< 20	< 20	< 20

* Normal rabbit serum

rocytes thereby rendering them agglutinable by K antibodies It should be mentioned briefly at this juncture that the presence of too much K antigen in the extract used for sensitization seemed to inhibit not only the K but also the O antigen HA reaction, particularly in the case of K51

In the case of K12 (Tables 1 and 4) there was a marked discrepancy between the bacterial agglutination and the HA tests in OK serum absorbed by homologous boiled culture while both reactions were positive in the OK serum absorbed by heterologous culture (Table 4) These results indicate that K12 contains at least two well developed K antigens, one of which gives positive BA and HA reactions while the other is responsible for the bacterial agglutination but does not fix readily to red cells If this last mentioned K antigen consists of protein it was not to be expected that red cells could be sensitized without pretreatment Table 5 shows the HA results using red cells treated with tannic acid prior to sensitization with K12 60°C extract The tanned cells gave a positive HAt test while the non treated cells did not react in the absorbed K12 serum At first this reaction was considered negative, until it was found that fixation of the antigen to the tannic acid treated cells was unstable being washed off from the red cell surface after more than two centrifugations

In the case of K51 and K52 no difference between the reaction of red cells before and after treatment with tannic acid was found

Escherichia coli O14 serum was also included in all HA tests, but HA reactions were negative in all cases Thus *Kunin*'s (14) common antigen (CA) was probably not involved in the present results

Double Diffusion in Gel and Immunoelectrophoresis

Irrespective of strain rabbit, temperature of extraction and test method, one very distinct precipitation line was found in the double gel diffusion experiments (a b and c of Figs 1 2 and 3) in all cases involving OK antisera These lines marked K were also present where O antibodies were absorbed with heterologous boiled culture In contrast the line could not be demonstrated after absorption with homologous boiled culture (Figs 1 and 2 a b c and Fig 3 a, b) No corresponding band was found in O antisera (Fig 3 c) although the 100°C extract formed this precipitate with OK antiserum (e g Fig 2 c)

Thus the line represents a thermostable K antigen It is thermostable in the sense that it can be demonstrated in the 100°C extract but it is non immunogenic since no antibody – K ' line – is present in the O antiserum No cross precipitations involving these K lines were seen among the three strains The results of the immuno-electrophoresis tests can be seen in d e f and g of Figs 1 2 and 3 What is said above about the K antigen is equally true here It can also be seen that the

laboratory strains may have lost L antigens during the years

One conclusion that can be drawn from these facts is therefore that a more precise definition of the K antigen in the single strains is needed This will probably only be possible if better methods for K determination are devised The bacterial agglutination technique should not be abandoned but be supplemented by others particularly by one of the precipitation methods

In order to reduce the number of changes in the existing nomenclature we would like to put forward the following proposal which is of course, open to discussion

The term K antigen should be preserved for all *surface antigens* except O and H To mark the general character of the polysaccharide K antigens which are by far the most important for classification they should keep the suffix (B) because these antigens fit best with the original description of the B antigen However the polysaccharide K antigen of those strains which have to be autoclaved to become agglutinable in O antiserum should still be called A We know now that several *E coli* strains contain thermostable K antigens (B) and that practically all former L test strains do so (16) In addition to the thermostable K antigen (B) many of these former L strains contain one or more thermolabile K antigens (L) As it seems to be easier to demonstrate and handle the K(B) antigens than the K(L) antigens the original K numbers of the L test strains should be attached to the thermostable antigen and not retained for the thermolabile antigens This proposal will cause some changes in the suffixes but will keep the established K numbers fixed to the test strains and serotypes to which they have always belonged

The antigens of the three strains examined in this paper would be expressed as follows

Su65/42	O4 K12(B)	K?(L)	H–
Al83a	O1 K51(B)		H–
Al0b	O4 K52(B)		H–

We would not propose at this juncture a new K number for the thermolabile K antigen in Su65/42 capable of coating tannic acid treated red cells but this must be done. We are also aware of the fact that additional K(L) antigens could perhaps be added to the seroformulas of all three strains. However these antigens have not yet been clearly defined

Finally it should be stressed here that the fundamental serological division among *E coli* strains is based on the O antigens If further subdivision is necessary the type of the K(B) polysaccharide antigens and the H antigens must be determined

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6 *Jann B & Jann K* Personal communication 1967

7 *Jann K Jann B Ørskov I Ørskov F & Westphal O* Immunchemische Untersuchungen an K Antigenen von *Escherichia coli* II Das K antigen von *E coli* O8 K42(A) H– Biochem Z *342* 1–22 1965

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9 *Jann K Jann B Ørskov F & Ørskov I* Immunochemistry of K antigens of *Escherichia coli* 5 The K antigen of *E coli* O8 K27(A) H– Europ J Biochem *5* 456–465 1968

shown to contain a thermostable, negatively charged K antigen In immuno-electrophoresis these extracts will show a thermostable antigen moving towards the anode i e exactly the behaviour one would expect from an acidic polysaccharide Here it can be mentioned that preliminary chemical investigations have shown that these K antigens can be separated by centrifugation from the O lipopolysaccharide extracted by the phenol water method They are polysaccharide in nature and hexuronic acids have not been found (6)

It is a well known fact that the lipopolysaccharide will hardly sensitize erythrocytes without heating or alkali treatment (15) while polysaccharide antigens will coat red cells without pretreatment (12) *Spaun* (21) determined the *Salmonella* Vi antigen by HA test and the capacity has been used extensively to demonstrate *E coli* K polysaccharide (e g 5)

In agreement with the idea that the heated and unheated extracts of K12 K51 and K52 contain polysaccharides such extracts coat sheep erythrocytes readily Using the HA technique (without tannic acid treatment) it could be shown that the L serum produced by absorption of the OK serum with homologous boiled culture did not contain detectable amounts of agglutinin against the K determinant

If the three K antigens which undoubtedly consist of polysaccharide were to be named they would probably be called B antigens (see later) However, what constitutes the L characters described previously? The three classical L strains examined here have antigenic determinants detectable by agglutination experiments in OK antisera absorbed by homologous boiled culture *Kauffmann* (10) had already noted that L titres c[illegible] be low and that rabbits differed much in [illegible]r capacity to produce L antibody He al[illegible]bserved that O inagglutinable strains ga[illegible] lower titre in the pure L serum than in [illegible] unabsorbed OK antiserum *Knipschildt* [illegible]) stated that the L antigen probably cont[illegible]ed both a thermostable and a thermolabile c[illegible]nstituent *Vahlne* (23), who carried out many K determinations used only unabsorbed OK antisera because of lack of media, and used the criterion of inagglutinability in O antiserum to secure his K determination What he detected was probably in most cases the thermostable K antigen described in this paper *Vahlne* wrote " as even the L antigen in spite of heating to 100°C is able in a slight degree to combine with antibodies through absorption Indeed the borders between the L and B antigens are fluctuating One of the three strains examined here the test strain for *E coli* K antigen 12 has a thermolabile antigen giving a high bacterial agglutination titre an antigen which will also coat tanned erythrocytes and might thus be a protein Other protein K antigens have been describ d but the only one which has been numbered is K88 which is a pure protein of fimbria like character (17 22) K88 is generally found in strains which in addition have polysaccharide K antigens It may also be mentioned that many coli strains have fimbriae that are proteinaceous and that such fimbriae may behave in the same way as L antigens It is a well known fact today that many *E coli* OK sera produced with strains subcultured exclusively on solid media will contain H antibodies Strains of this sort might be misinterpreted as containing K(L) There are probably many other unknown thermolabile surface constituents which can play the role of the L antigen Finally one more possibility should be mentioned In the typical B antigen strain heating to 100°C for 1 hour will leave sufficient B antigen on the bacterial surface to absorb all B agglutinins from the OK serum Only small amounts of thermostable K antigen are left on the surface in some L strains When the OK serum is absorbed the K titre will be lowered but not eliminated In some such cases repeated absorption will completely eliminate agglutinins against the thermostable antigen This is probably the situation with quite a number of the classical L strains Such strains might easily be called B in one laboratory and L in another It should be added that some old

laboratory strains may have lost L antigens during the years

One conclusion that can be drawn from these facts is therefore that a more precise definition of the K antigen in the single strains is needed This will probably only be possible if better methods for K determination are devised The bacterial agglutination technique should not be abandoned but be supplemented by others particularly by one of the precipitation methods

In order to reduce the number of changes in the existing nomenclature we would like to put forward the following proposal which is of course open to discussion

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The antigens of the three strains examined in this paper would be expressed as follows

Su65/42	O4 K12(B)	K?(L)	H–
A183a	O1 K51(B)		H–
A10b	O4 K52(B)		H–

We would not propose at this juncture a new K number for the thermolabile K antigen in Su65/42 capable of coating tannic acid treated red cells but this must be done We are also aware of the fact that additional K(L) antigens could perhaps be added to the seroformulas of all three strains However these antigens have not yet been clearly defined

Finally it should be stressed here that the fundamental serological division among *E coli* strains is based on the O antigens If further subdivision is necessary, the type of the K(B) polysaccharide antigens and the H antigens must be determined

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test and the Kunitz method when testing the interaction between some commercially avail able enzymes and inhibitors The CPI test was considered to be of value, especially when working with inhibitors and enzymes of low activity and when screening the effect of many different inhibitors against various en zymes The aim of the present work was to study by this method the inhibitory effect of sera from different species against some pro teolytic enzymes of animal microbial, and plant origin, and to compare the inhibitory spectrum and complexity of the different sera

MATERIALS AND METHODS

Enzymes

The following enzymes were obtained from Sigma* Trypsin from bovine pancreas (Type III 2 x crystallized lot 97B 8000) α chymotrypsin from bovine pancreas (Type II 3 x crystallized lot 86B 0470) protease from *Bacillus subtilis* (Type VIII crystallized lot 17B 2770) protease from *Aspergillus oryzae* (Type II crude lot 1320) ficin from fig tree latex (2 x crystallized lot 119B-4750) papain from papaya latex (2 × crystallized lot 47B 1020) Trypsin from hog pancreas (crystallized batch No 36467) was obtained from Koch Light Laboratories Ltd **

The other microbial enzymes used were usually prepared in one of the following ways a) Culture filtrates or liquid cultures were preserved by the addition of merthiolate to a final concentration of 1 10000 without further purification or concentration b) Liquid cultures were centrifuged the enzymes present in the clear liquid concentrated by salting out with ammonium sulphate to 60 per cent saturation the precipitate collected and dialysed against distilled water and preserved by addition of merthiolate c) The organisms were cultured on the surface of nutrient agar with or without skim milk (10–15 per cent) added The cultures were frozen after a period of incubation thawed the liquid pressed out through sterile gauze and then treated as described under method b d) The microorganisms were inoculated in 30 ml of saline on to the surface of 50 ml of a 2 per cent nutrient agar in 500 ml Erlenmeyer flasks the flasks were incubated in a rotatory shaker and the enzymes collected from the supernatant after centrifugation usually by salting out with ammonium sulphate The time of incubation varied from 16 hours (*Staphylococcus aureus*) to 7 days (*Corynebacterium pyogenes*) and the incubation temperature from 20 to 37 C In each case the medium and other conditions giving the highest yield of enzyme were chosen

The enzyme solutions were usually stored at + 4 C at which temperature most of the proteinases were fairly stable Many of the diluted enzymes used in the CPI test could be stored for several weeks at + 4 C without any considerable loss of activity Merthiolate was added to the solutions to a final concentration of 1 10000 The strength of the enzyme solutions used in the CPI test was usually of the order of 100 to 1000 CP units per 0 025 ml (*Fossum 1970*) In the Kunitz method an amount of enzyme which in the absence of inhibitors gave an increase of OD value in the supernatant of about 0 600 was added to each tube In the case of ficin and papain sulfhydryl activators were always added to the enzyme solutions

Blood Sera

The whole blood samples were allowed to coagulate and the serum was collected after centrifugation Assays for inhibitors were usually performed on fresh sera When preliminary studies had shown that storage at low temperatures did not influence the inhibiting capacity sera stored in a refrigerator or frozen at –22 C were sometimes used In the case of rabbit serum especially the inhibitory capacity was reduced considerably after a few days storage at room temperature and even at +4 C after some weeks while bovine inhibitors proved to be more stable Thus bovine sera stored at room temperature for five years still exhibited an appreciable inhibitory activity Merthiolate was usually added to the sera to a final concentration of 1 10000 As a rule sera from several individuals of the same species were investigated including young and older animals

Determination of Inhibitors

The Kunitz method for the determination of inhibitors (*Kunitz 1947*) was performed with the modification described by *Fossum (1970)* For trypsin parallel experiments with sera from at least two individuals of the same species were carried out For the other enzymes usually one serum from each species was used With regard to rabbit a great number of sera from different individuals were investigated

The casein precipitating inhibition test (CPI test) was carried out as described by *Fossum* (1970) using as substrate sodium caseinate incorporated in agar The CPI test was performed for the qualitative and semiquantitative determin-

* Sigma Chemical Company St Louis Mo USA
** Koch Light Laboratories Ltd Colnbrook Buckinghamshire England

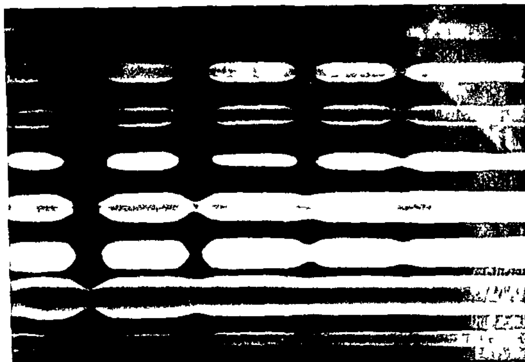


Fig 1 Crosswise CPI test with different dilutions of human serum The dilutions are (from left to right as marked by arrows) 1 1 1 10 1 50 1 100 and 1 500 Enzymes are (downwards) Bovine trypsin (0 01 mg per ml) swine trypsin (0 005 mg per ml) α chymotrypsin (0 005 mg per ml) proteinases from *Bacillus subtilis* (0 001 mg per ml) *Aspergillus oryzae* (0 03 mg per ml) and *Bacillus cereus* ficin and papain (0 05 mg protein per ml)

ation of inhibitors in a serum sample by placing filter paper strips moistened with different dilutions of serum on the agar surface and afterwards strips with enzyme solutions at right angles to the inhibitors Inhibition occurred as interruptions of the otherwise white lines of precipitation or by a more or less narrowing of these lines (Figure 1) The test is referred to as the *crosswise CPI test*

Electrophoretic Differentiation of the Serum Inhibitors

The sera were separated by paper electrophoresis before being brought into contact with the enzymes An electrophoresis apparatus 3276 BV LKB Stockholm was used with Schleicher and Schull filter paper No 2043 bmgl (40 x 410 mm) Phosphate buffer 0 05M pH 6 2 with merthiolate to a final concentration of 1 10000 was used The sera were applied in amounts of 8 – 12μl and subjected to electrophoresis at 120 V for 16 – 18 hours The wet paper strips were then transferred immediately to the surface of the caseinate medium After incubation at 37 C for 2–3 hours the strips were removed from the medium and replaced by narrow (4 mm) strips of filter paper which were moistened with solutions of the enzymes to be tested Usually 3 filter paper strips were placed in parallel within the 4 cm broad field of the electrophoresis paper for 5–24 hours at 37 C Precipitation zones occurred along the enzyme containing strips (Figs 2–6) Inhibition is indicated by interruption of the white precipitation zones or by a narrowing of the precipitation zones depending upon the inhibitory activity and the enzyme concentration The normal inhibitors were localized on the anode side of the line of application The combination of electrophoresis of inhibitor-containing material and the use of the CPI test is referred to as the *electrophoretic CPI test*

RESULTS

Assay of the Inhibitory Activity of Different Sera upon Proteolytic Enzymes Based on the CPI Test

Fig 1 shows the inhibitory activity of different dilutions of human serum upon e

TABLE 1 *Highest Dilution of Serum from Various Species Resulting in Inhibition of Different Proteolytic Enzymes The Data are Based upon the Crosswise CPI Test*

Sera	*Proteolytic enzymes origin* Swine trypsin	α Chymo trypsin	*Bacillus subtilis*	*Aspergillus oryzae*	*Bacillus cereus*	*Staphylo coccus aureus*	*Pseudomonas aeruginosa*	Ficin papain
Man	1 500*	1 500	1 500	1 200	1 200	1 100	1 500	1 100
Pig	1 200	1 100	1 200	1 200	1 200	1 200	1 500	1 50
Cattle	1 500	1 500	1 100	1 100	1 500	1 200	1 500	1 100
Sheep	1 500	1 500	1 200	1 200	1 500	1 100	1 500	1 100
Goat	1 500	1 500	1 500	1 200	1 500	1 100	1 500	1 100
Horse	1 500	1 500	1 500	1 50	1 500	1 50	1 500	1 50
Rabbit	1 200	1 100	1 100	1 100	1 100	1 10	1 100	1 50
Dog	1 100	1 100	1 10	1 10	1 50	1 10	1 50	1 50
Hen	1 200	1 100	1 200	1 200	1 50	1 50	1 50	1 10

* The sera were diluted 1 10 1 50 1 100 1 200 1 500 and 1 1000 in saline

different proteolytic enzymes It can be seen that, up to a serum dilution of 1 100 all the enzymes are inhibited to some extent while at the dilution 1 500 a weak inhibition can only be observed for swine trypsin α chymo trypsin and *Bacillus subtilis* proteinase In Table 1 the inhibitory activity of sera from different animal species upon different proteo lytic enzymes *is shown* The sera of man, cattle, sheep goat and horse inhibit trypsin and most of the other proteolytic enzymes to a greater extent than do the sera of pig, rab bit dog and hen Of the microbial enzymes tested the proteinase from *Staphylococcus aureus* is inhibited to a less extent than the others Other bacterial proteinases not shown in the table including those of *Corynebacte rium pyogenes Serratia marcescens Proteus vulgaris Pseudomonas fluorescens* and a strain of *Staphylococcus epidermidis* were strongly inhibited

Demonstration of Zones of Inhibition by the Electrophoretic CPI Test

Fig 2 shows the electrophoretic patterns of the inhibitors in human serum against six different proteolytic enzymes Two zones of inhibition are seen for swine trypsin α chymo trypsin and the proteinases from *Bacillus sub ti s* and *Aspergillus oryzae* while e protein as n *acillus cereus* and *P udomonas* v only one zone c inhibition

It can also be seen that the slow moving factor inhibits trypsin, α chymotrypsin, and proteinase from *Bacillus subtilis* only to a small extent compared with the inhibition caused by the fast moving inhibitor The pro teinases from *Bacillus cereus* and *Pseudomo nas aeruginosa* are inhibited only by the slow moving inhibitor By diluting the serum 1 5 and 1 10 before carrying out electrophoresis the inhibitory activity of the slow moving in hibitor upon trypsin and chymotrypsin could barely be demonstrated although the inhibi tion upon the *Bacillus cereus* proteinase was still evident

The patterns of proteolytic inhibitors in sera from pig cattle and rabbit are shown in Figs 3 4 and 5 respectively In some cases two or more zones of inhibition are confluent By diluting such a serum before carrying out electrophoresis or by increasing the strength of the developing enzyme the presence of two zones of inhibition representing at least two inhibitors could be demonstrated Thus it was indicated that the slow moving tryp in inhibitor in cattle serum migrats like the α chymotrypsin inhibitor under the condi tions used (Table 2) Fig 6 shows the in hibitory spectrum of bovine serum diluted 1 5 before electrophoresis was carried out upon swine trypsin α-chymotrypsin and proteinase from *Bacillus cereus* It is evident that the prominent inhibition of cattle serum upon

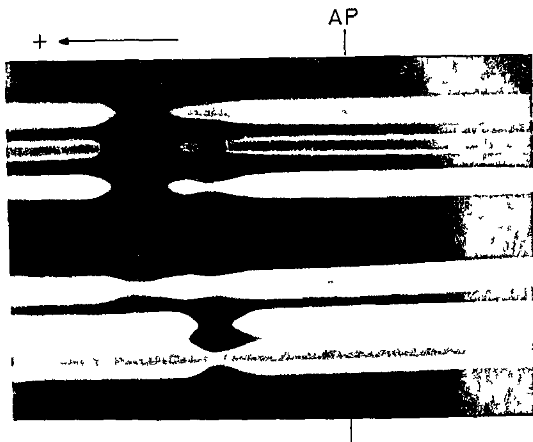


Fig 2 Electrophoretic CPI test of human serum The enzymes used are (downwards) Swine trypsin α-chymotrypsin proteinases from *Bacillus subtilis Aspergillus oryzae Bacillus cereus* and *Pseudomonas aeruginosa* The naturally occurring inhibitors are situated on the anode side of the line of application (AP) The electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 hrs at 120 V

these three enzymes is caused by at least three different inhibitors with different migration rates

The spectra of inhibitors in sera of various species upon different proteolytic enzymes are indicated in Tables 2 and 3 The data given are based upon electrophoresis of whole sera and different dilutions of the sera as well as development with enzyme solutions of various strengths It should be pointed out that dog sera from different breeds (English setter vorster poodle) seemed to vary somewhat as to the patterns of inhibitors but further investigation has not been carried out on this question The strength of the fast moving trypsin inhibitor from different individuals of rabbit also varied to a great extent

The zones of inhibition for ficin and papain were usually much weaker than for the other enzymes The inhibitory spectrum of the sera tested was equal for these two enzymes

The inhibitory spectra of sera from different species upon three different enzymes are shown in Table 4 Electrophoresis was carried out with different dilutions of the sera These results give some information as to the relative strengths of the inhibitors present in each serum

In some cattle and pig sera, especially from older individuals a zone of inhibition was found near the line of application for some microbial enzymes Of the microbial en[illegible]me tested the proteinases from *C*[illegible] *pyogenes Bacillus subtilis* [illegible]

TABLE 3 *The Patterns of Inhibitors in Sera from Rabbit Dog and Hen upon Different Proteolytic Enzymes Based on the Electrophoretic CPI Test*

Proteolytic enzymes (Origin)	Sera									
	Rabbit				Dog			Hen		
	F*	I₁	I₂	S	F	I	S	F	I	S
Swine trypsin	+ + + +		+ + + +		+ + + +		+ + +	+ + +	+ +	+ + + +
α Chymotrypsin	+ + +	+ + +	+ + +		+ + + +		+ + +	+ + + +		+ + +
Bacillus subtilis	+ + +	+ + +		+	+ +	+ +			+ +	+ + + +
Aspergillus oryzae	+ +	+ +			+ + +	+ +		+	+ +	+ + + +
Bacillus cereus	+ + + +			+ +	+ +	+			+ + + +	
Pseudomonas aeruginosa	+ + + +			+ +	+ +	+ +			+ + + +	
Proteus vulgaris	+ +			+ +	+ +	+ +		+ +	+ +	
Staphylococcus aureus	+ +			+	+ +	+				+
Corynebacterium pyogenes	+ +			+ +	+ +	+ +		+ +	+ + +	
Ficin	+			+	+		+			+
Papain	+			+	+		+			+

* The same symbols as in Table 2 In case of rabbit serum two intermediary inhibitors were demonstrated these were designated I_1 and I_2

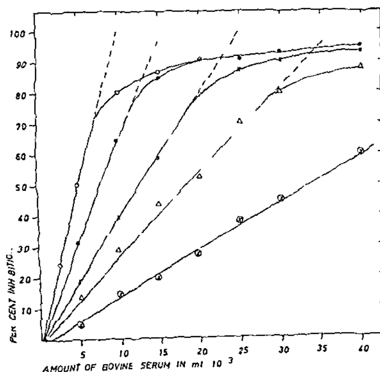


Fig 7 Per cent inhibition of bovine trypsin (×—×) α chymotrypsin (●—●) proteinases from *Pseudomonas aeruginosa* (○—○) and *Bacillus subtilis* (⊗—⊗) and ficin (△—△) by bovine serum The amounts of enzymes used were those giving an increase in OD value of about 0 600 in the absence of inhibitor i e 0 0 5 mg of bovine trypsin 0 0125 mg of α chymotrypsin and 0 00625 mg of ficin

shown whether the factors responsible for the inhibition of proteinases from *Bacillus subtilis* and *Aspergillus oryzae* are identical with the trypsin and chymotrypsin inhibitors and whether the slow moving inhibitor affecting the other microbial enzymes is identical with the α trypsin inhibitor The possibility that the inhibition of several proteinases may be due to the same substance is supported by the similarity in electrophoretic mobility as well

TABLE 4 *Inhibitory Patterns of Dilutions of Sera from Different Species for Swine Trypsin α Chymotrypsin and Proteinase from* Bacillus cereus *The Data are Based upon Results Obtained by the Electrophoretic CPI Test of the Diluted Sera*

Sera	Dilution	Trypsin	α Chymotrypsin	*Bacillus cereus proteinase*
Man	1 2	F* S	F S	S
	1 5	F S	F S	S
	1 10	F –†	F –	S
	1 30	F –	F –	–
Pig	1 2	F S	F S	S
	1 5	F S	F S	S
	1 10	– S	F –	S
	1 30	– –	– –	–
Cattle	1 2	F I	I	S
	1 5	F I	I	S
	1 10	F I	I	S
	1 30	F –	I	S
Sheep	1 2	F S	F S	S
	1 5	F S	F S	S
	1 10	F –	F –	S
	1 30	F –	F –	S
Goat	1 2	F S	F S	S
	1 5	F S	F S	S
	1 10	F S	F S	S
	1 30	F –	F –	S
Horse	1 2	F S	F S	S
	1 5	F S	F S	S
	1 10	F S	F S	S
	1 30	F S	F S	S
Rabbit	1 2	F I	F I	F S
	1 5	F I	F I	F S
	1 10	F I	– –	– –
	1 30	– –	– –	– –
Dog	1 2	F S	F S	F I
	1 5	F –	F –	F –
	1 10	– –	F –	– –
	1 30	– –	– –	– –
Hen	1 2	F I S	F S	I
	1 5	– – S	F –	I
	1 10	– – –	– –	I
	1 30	– – –	– –	–

* The same symbols as in Table 2
† No demonstrable inhibition

as the fact that purified inhibitors have been found to inhibit a number of proteolytic enzymes to various degrees. However the existence of more than the one or two inhibitors found here cannot be ruled out. Firstly one zone of inhibition can be caused by more than one inhibitor This was often the case when undiluted serum was used but after dilution two zones of inhibition occurred Secondly different inhibitors can have the same migrating rate Thirdly the unknown inhibitor(s) can be so weak that they will not be detected by the method used The inability to distinguish the trypsin inhibitor lying between the α_1 and α globulin fractions and the chymotrypsin inhibitor in the postalbumin fraction may be due to one or more of the afore mentioned explanations The fact that the inhibition of microbial enzymes is mainly caused by the slowly migrating inhibitor fraction is of interest as it has been discussed whether bacterial enzymes in the case of α_1 trypsin deficiency in human may be responsible for the destruction of pulmonary parenchyma (*Miller & Kuschner* 1969)

So far the inhibitors in animal sera have not been investigated to the same extent as those in human serum From Table 2 and 3 and Figs 3 4 5 and 6 it can be seen that all the animal sera examined contain two inhibitors for trypsin and usually two for α chymotrypsin and the proteinases from *Bacillus subtilis* and *Aspergillus oryzae* *Nakamura & Wakeyama* (1961) found by two dimensional paper electrophoresis two inhibitors for trypsin in sera from man horse sheep and rabbit while they were able to demonstrate only one trypsin inhibitor in the

TABLE 5 *Amount of Bovine Trypsin α Chymotrypsin and Ficin Inhibited by 1 ml of Sera from Different Species*

Sera	Amount of enzymes inhibited (mg) Bovine trypsin	α Chymotrypsin	Ficin
Human	1 – 1 2	0 8	0 14
Pig	0 6 – 1 0	0 3	0 08
Cattle	1 0 – 1 6	0 8	0 16
Sheep	1 2 – 1 6	0 8	0 16
Goat	1 2 – 1 6	0 6	0 16
Horse	1 2 – 1 6	0 6	0 16
Rabbit	0 4 – 0 8	0 3	0 11
Dog	0 4 – 0 5	0 3	0 11
Hen	0 6 – 0 8	0 3	0 06

TABLE 3 *The Patterns of Inhibitors in Sera from Rabbit Dog and Hen upon Different Proteolytic En ymes Based on the Electrophoretic CPI Test*

Proteolytic enzymes (Origin)	Sera									
	Rabbit				Dog			Hen		
	F*	I_1	I_2	S	F	I	S	F	I	S
Swine trypsin	+ + + +		+ + + +		+ + + +		+ + +	+ + +	+ +	+ + + +
α Chymotrypsin	+ + +	+ + +	+ + +		+ + + +		+ + +	+ + + +		+ + +
Bacillus subtilis	+ + +	+ + +		+	+ +	+ +			+ +	+ + + +
Aspergillus oryzae	+ +	+ +			+ + +	+ +		+	+ +	+ + + +
Bacillus cereus	+ + + +			+ +	+ +	+			+ + + +	
Pseudomonas aeruginosa	+ + + +			+ +	+ +	+ +			+ + + +	
Proteus vulgaris	+ +			+ +	+ +	+ +		+ +	+ +	
Staphylococcus aureus	+ +			+	+ +	+				+
Corynebacterium pyogenes	+ +			+ +	+ +	+ +		+ +	+ + +	
Ficin	+			+	+		+			+
Papain	+			+	+		+			+

* The same symbols as in Table 2 In case of rabbit serum two intermediary inhibitors were demonstrated these were designated I_1 and I

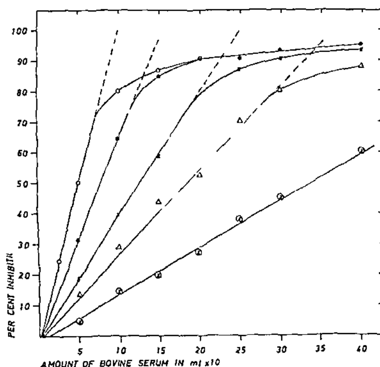


Fig 7 Per cent inhibition of bovine trypsin (×—×) α chymotrypsin (●—●) proteinases from *Pseudomonas aeruginosa* (○—○) and *Bacillus subtilis* (⊗—⊗) and ficin (Δ—Δ) by bovine serum The amounts of enzymes used were those giving an increase in OD value of about 0 600 in the absence of inhibitor i e 0 025 mg of bovine trypsin 0 0175 mg of α chymotrypsin and 0 00625 mg of ficin

shown whether the factors responsit for the inhibition of proteinases from *Bacill subtilis* and *Aspergillus oryzae* are identical ith the trypsin and chymotrypsin inhibi s and whether the slow moving inhibitor ffecting the other microbial enzymes is identical with the α trypsin inhibitor The possibility that the inhibition of several proteinases may be due to the same substance is supported by the similarity in electrophoretic mobility, as well

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NON-PHOTOCHROMOGENIC MYCOBACTERIA SEROTYPE DAVIS

The Inhomogeneity within the Serological Group and the Relationship to Mycobacterium avium

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Non photochromogenic mycobacteria belonging in serotype Davis deviate from the typical pattern of *M avium* The divergencies in the *in vitro* experiments occurred especially in connection with drug resistance effect of temperature and catalase test In the *in vivo* examinations there were divergencies in all the animal studies The Davis strains are unstable in subcultures on Lowenstein Jensen and 7H 10 agar media and pure cultures of smooth transparent colonies (light) were dominated by opaque variants (dark) after 7–8 passages It is probable that the variants are the chief reason for the inhomogeneity within the Davis group On the basis of virulence experiments the strains were divided into three groups The most virulent (Group A virulent for hens rabbits guinea pigs) gave growth of light colonies and the attenuated (Group C) dark colonies However the correlation between the two characteristics is not absolute since both types of colonies were represented in pure culture in Group B (virulent for hens and guinea pigs attenuated for rabbits) i e also the strains which give growth of dark colonies may possess virulence for the classical experimental animal the hen In the *in vitro* experiments the attenuated strains (Group C) were more sensitive to streptomycin viomycin rifampicin and penicillin than the strains in the other groups This greater sensitivity of the variants must be taken into consideration when selecting test strains for assay of the effect of new drugs or drug combinations

A previous publication (5) gave a report on a number of virulent strains of *M avium* isolated from Danish patients One third of these strains could be classified as serotype Davis Furthermore an analysis of the whole material from animals (4) and man (5) showed some distinctive characterization of serotypes Davis and Av II The material of serotype Davis strains was small and had been selected on the basis of virulence studies. Therefore only a limited description could be given of strains belonging in that serotype

In the present work, the material is supplemented by Davis strains received from abroad Tendency to produce variants during subculture was confirmed with these strains and relationships were demonstrated between colony morphology differences in virulence and drug resistance pattern It was particularly the results in these experimental spheres that were responsible for the inhomogeneity within the Davis group and which also affected the number of divergencies from the typical pattern of *M avium*

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Fig 5 Three week old rough colony Davis strain on 7H 10 agar Magnification 100 ×

The significance of the number of passages on the two media before changes occurred in the colony morphology was examined in passages 2–9 by streaking a representative mass of culture on 7H 10 agar plates Unchanged conditions were seen in the first three to four passages after which increasing numbers of dark colonies appeared which completely predominated from the seventh and eighth passages

A few rough colonies were found with both strains as early as the first passage However, they did not appear systematically in subsequent passages and were always small in num

TABLE 6 *Davis Strains Drug Resistance*

Drug	No of strains	Code for concentration of drug**						No of divergences from *M avium* pattern
		0	1	2	3	4	5	
PAS	19	0	5*	0	3	11		5
TSC	19	0	4*	2*	2	11		6
INH	19	0	0	3*	16*	0	0	19
Cycloserine	19	18	1	0	0	0		0
Erythromycin	12	6	3	3	0			

** *Code for concentration of drug γ/ml*

	0	1	2	3	4	5
PAS	0	0.4	1.6	6.4	25.6	
TSC	0	0.4	1.6	6.4	25.6	
INH	0	0.08	0.32	1.28	8.0	50.0
Cycloserine	0	32.0	64.0	128.0	256.0	
Erythromycin	0	1.0	5.0	10.0		

* = divergencies

TABLE 7 *Davis Strains Drug Resistance*

Drug	Group	No. of strain	Code for concentration of drug** 0	1	2	3	4	No. of divergencies from *M. avium* pattern
Streptomycin	A + B	12	1*	0	0	6	5*	6
	C	7	2*	0	3	2	0	2
Viomycin	A + B	12	0	0	2	10	0	0
	C	7	0	3	3	1	0	0
Rifampicin	A + B	6	0	0	2	4	0	
	C	7	5	2	0	0	0	
Penicillin	A + B	12	1	1	10			
	C	7	4	3	0			

** *Code for concentration of drug γ/ml*

	0	*1*	*2*	*3*	*4*
Streptomycin	0	2.0	4.0	16.0	64.0
Viomycin	0	10.0	40.0	160.0	640.0
Rifampicin	0	16.0	32.0	64.0	128.0

Penicillin 0 = growth on control tube only
1 = few colonies on 100 γ
2 = growth on 100 γ same as control tube

* = divergencies

ber The rough variants observed in the present experiments occurred only in the passages on 7H 10 agar medium

In the subsequent presentation of the results division of the material into the three groups is relevant only in connection with the determination of drug resistance where there were definite differences between the strains All the other results are given collectively for the whole material

Colony morphology on Lowenstein Jensen medium The colonies were relatively uniform as regards morphology being moderately eugonic dome shaped and smooth SSC 1169 contained colonies of different sizes

Colony pigmentation All strains gave growth of pale yellow colonies on Lowenstein Jensen medium Short exposure to light had no effect while long exposure increased the colour in all cases

Drug resistance The results of determination of the resistance of the strains to PAS TSC INH cycloserine and erythromycin are shown collectively for the whole material in Table 6 The results with streptomycin viomycin rifampicin and penicillin are given separately in Table 7 for the three groups since there was a marked tendency to the greatest sensitivity of the attenuated strains (Group C)

The number of divergencies from the typical pattern of *M avium* are shown in the tables It will be seen that apart from streptomycin these divergencies are due to Davis strains being more sensitive than *M avium* The number of divergencies i about two per strain Such comparison has not been made with erythromycin rifampicin and penicillin

Effect of temperature on growth on Lowenstein Jensen medium The results of examination of this aspect are shown in the column diagram in Fig 6 where the values refer to the first time (in weeks) at which well isolated colonies were visible It will be seen that practically all the strains grew in the course of one week at 37° 40° 43° and 45°, while growth at 22° was somewhat slower This relatively rapid growth of Davis strains gives about four divergencies per strain from the typical pattern of *M avium*

Comparison of the number of col eight weeks showed identic germination at 37° and 4



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Fig 5 Three week old rough colony *Davis strain* on 7H 10 agar Magnification 100 ×

The significance of the number of passages on the two media before changes occurred in the colony morphology was examined in passages 2–9 by streaking a representative mass of culture on 7H 10 agar plates Unchanged conditions were seen in the first three to four passages after which increasing numbers of dark colonies appeared which completely predominated from the seventh and eighth passages

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TABLE 6 *Davis Strains Drug Resistance*

Drug	No of strains	Code for concentration of drug**						No of divergencies from M *avium* pattern
		0	1	2	3	4	5	
PAS	19	0	5*	0	3	11		5
TSC	19	0	4*	2*	2	11		6
INH	19	0	0	3*	16*	0	0	19
Cycloserine	19	18	1	0	0	0		0
Erythromycin	12	6	3	3	0			

** *Code for concentration of drug γ/ml*

	0	*1*	*2*	*3*	*4*	*5*
PAS	0	0.4	1.6	6.4	25.6	
TSC	0	0.4	1.6	6.4	25.6	
INH	0	0.08	0.32	1.28	8.0	50.0
Cycloserine	0	32.0	64.0	128.0	256.0	
Erythromycin	0	1.0	5.0	10.0		

* = divergencies

The potential significance of this observa tion warranted a more extensive evaluation of the PHA response of the spleen cells from hypogammaglobulinaemic bursectomized irra diated chickens In the present communica tion the optimal conditions for the PHA res ponse of spleen cells in tissue culture were first defined The PHA induced increases of the synthesis of DNA RNA and protein were then followed during a 48 hour period The dynamics and magnitudes of the responses of spleen cells from hypogammaglobulinaemic bursectomized irradiated and normogamma globulinaemic control irradiated chickens were compared

MATERIALS AND METHODS

Animals 8 weeks old White Leghorn cockerels of the non inbred De Kalb chick strain 161 (Mun roe Hatchery Joliet Ill) were used They were raised from newly hatched chickens in conventional animal quarters

Bursectomy and X irradiation Surgical bursec tomies were performed on the day of hatching as previously described (16) The animals including the unoperated were x irradiated on the same day The dosage was 600 R under conditions described in detail elsewhere (1)

Immunoglobulin assays Heparin zed plasma samples were obtained from the chickens at 7 v eeks of age They were analysed semiquantitatively for content of IgG and IgM as previously described (1) Only bursectomized irradiated (Bx) chickens lacking detectable immunoglobulin levels v ere in cluded in this investigation Control irradiated (Cx) animal had normal immunoglobulin levels

Preparation of spleen cell suspensions The chick ens were sacrificed by bleeding from the heart The pleens were dissected out minced with fine scis-sors and gently passed through a 60 mesh stainle s steel screen Dulbecco s phosphate buffered saline (PBS) supplemented with 5 per cent (v/) heat inactivated (56 for 30 minutes) foetal calf serum (FCS) (Colorado Serum Company Denver Co lo) was continuously added The spleen cells were further dispersed by gently passing through a 22 gauge needle into a 50 ml disposable plastic cul ture tube (Falcon Plastics Los Angeles Calif) Large cell aggregates were allov ed to settle for 5 minutes The supernatant v as collected washed 3 x with PBS FCS and resu pended in Eagle s Mini mum Essential Medium (morolayer modification) containing ? mM/ml L glutamine 100 units/ml penicillin 100 μg/ml streptomycin (Grand Island Biological Grand Island N Y) and supplemented with 10 per cent FCS L leucine was excluded from the medium when the H^3 L leucine incorporation was studied

Cell counts were performed using the Natt Her rick stain (ↄ) and the cell suspension v as diluted to desired cell concentration with the complete medium

Cotton column filtration The spleen cell suspen sion was prepared as described above but the PBS contained 50 per cent (v/v) autologous fresh hep arinized (20 U/ml) chicken plasma and 1 per cent (w/v) dextran m.w 250 000 (Pharmacia Upp sala Sweden) instead of FCS The spleen cell sus pension was poured onto a column containing an equal volume of cotton wool (Red Cross Cotton Johnson and Johnson New Brunswick N J) in cubated for 15 minutes at 37 C and then eluted with two volumes of the PBS chicken plasma dex tran solution.

The cell suspension was then centrifuged and the cells were washed 3 x v ith PBS FCS and finally suspended in complete tissue culture me dium

Tissue culture 1 ml volumes of the cell suspen sion were added to 16 x 100 mm dis posable flint glass ti sue culture tubes (Bellco Glass I ı Vine land N J) The tubes were maintained v ith loose ly fitted plastic caps at 40 C in a moi t ↄ per cent CO_2–95 per cent air atmosphere

Phytohaemagglutinin (PH 1) Wellcome phyto haemagglutinin (Burroughs Wellcome and Co London England) was reconstituted with ↄ ml of PBS per vial and add d in indicated volumes to the cultures

Assay procedures

DNA synthesis The incorporation of H^3 methyl thym dine of the cultured cells during a 4 hour period was taken as a measurement of the rate of DNA synthesis of the cultures and was used as a general indicator of the re ponse of cells to PHA when suitable tissue culture condit ons we e estab lished 1 μc of H^3 methylthymidine specific activi ty 6 7 c/mM (New England Nu lear Corp Boston Mass) in 0 050 ml of PBS was added per culture tube After 4 hours the tubes v ere chilled and cen trifuged at 270 x g for 10 minutes at 4 C The supernatants were discarded The ell pellets were frozen and thawed once precipitated and extracted twice ith cold 5 per cent (w/) trichloro-acetic acid (TCA) and absolute methanol re pectively

RNA synthesis The incorporation of H^3 5 uri dine during a 15 minute and a 4 hour period was taken as an indicator of the rate of RNA synthesis The short pulse was used in an attempt to measure preferentially rapidly labelled RNA 5 μc H^3 5 uridine specific activity 4 c/mM (Schwarz Bic research Orangeburg N Y) in 0 050 ml of PBS was added per culture tube The horter pulse was

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IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS

1 *Phytohaemagglutinin Induced DNA RNA and Protein Synthesis in Spleen Cells from Control Irradiated and Bursectomized Irradiated Chickens*

GUNNAR V ALM

The Variety Club Research Center of the La Rabida University of Chicago Institute University of Chicago

Suitable conditions for the response of chicken spleen lymphoid cells to phytohaemagglutinin (PHA) in short term tissue culture were established The magnitudes and dynamics of the PHA induced changes in total DNA RNA and protein synthesis during a 48 hour period were followed The rate of the incorporation of H^3 uridine into RNA and H^3 L leucine into protein accelerated during the first 4 hours after the addition of the PHA The increase was linear for 24 and 36 hours respectively The increase of the incorporation of H^3 thymidine into DNA started at 16 hours and reached a maximum at 36 hours Spleen lymphoid cells from hypogammaglobulinaemic bursectomized irradiated and normogammaglobulinaemic control irradiated chickens responded in all but one respect equally well to PHA The increase of the rate of H^3 uridine incorporation determined by 4 hour pulses was more rapid for the spleen cells from bursectomized irradiated animals

The work of *Glick et al* (9) first indicated the role of the bursa of Fabricius in the devel[opment] of the antibody producing capacity of the chicken It is now well established that surgical removal of this lymphoid organ of the hindgut in the immediate period after hatching when followed by sublethal x irradiation drastically reduces antibody responses in later life and in a significant number of animals causes a severe hypogammaglobulinaemia (1 6 7) While immunoglobulin and specific antibody producing lymphoid cells are absent (1 6) the bursectomized irradiated hypogammaglobulinaemic chicken have normal numbers of small lymphocytes (6) *In vivo* manifestations of cellular immunity such as delayed hypersensitivity to diphteria toxoid graft versus host reactions and first set homograft rejections have been reported to be normal in such animals (6)

The *in vitro* response of spleen cells from hypogammaglobulinaemic bursectomized irradiated chickens to the mitogen phytohaemagglutinin as determined by the increase in the synthesis of DNA is also normal (1 6 13 16) although this cell population in the hypogammaglobulinaemic bursectomized irradiated chicken lack cells synthesizing immunoglobulin and specific antibody (1) This indicates a dissociation between the ability of chicken lymphoid cells to respond to phytohaemagglutinin (PHA) and to produce antibody

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The potential significance of this observation warranted a more extensive evaluation of the PHA response of the spleen cells from hypogammaglobulinaemic bursectomized irradiated chickens In the present communication the optimal conditions for the PHA response of spleen cells in tissue culture were first defined The PHA induced increases of the synthesis of DNA RNA and protein were then followed during a 48 hour period The dynamics and magnitudes of the responses of spleen cells from hypogammaglobulinaemic bursectomized irradiated and normogammaglobulinaemic control irradiated chickens were compared

MATERIALS AND METHODS

Animals 8 weeks old White Leghorn cockerels of the non-inbred De Kalb chick strain 161 (Munroe Hatchery Joliet Ill) were used They were raised from newly hatched chickens in conventional animal quarters

Bursectomy and X irradiation Surgical bursectomies were performed on the day of hatching as previously described (16) The animals including the nonoperated were x irradiated on the same day The dosage was 600 R under conditions described in detail elsewhere (1)

Immunoglobulin assays Heparinized plasma samples were obtained from the chickens at 7 weeks of age They were analysed semiquantitatively for content of IgG and IgM as previously described (1) Only bursectomized irradiated (Bx) chickens lacking detectable immunoglobulin levels were included in this investigation Control irradiated (Cx) animals had normal immunoglobulin levels

Preparation of spleen cell suspensions The chickens were sacrificed by bleeding from the heart. The spleens were dissected out minced with fine scissors and gently passed through a 60 mesh stainless steel screen Dulbecco s phosphate buffered saline (PBS) supplemented with 5 per cent (v/v) heat inactivated (56 for 30 minutes) foetal calf serum (FCS) (Colorado Serum Company Denver Colo) was continuously added The spleen cells were further dispersed by gently passing through a 22 gauge needle into a 50 ml disposable plastic culture tube (Falcon Plastics Los Angeles Calif) Large cell aggregates were allowed to settle for 5 minutes The supernatant was collected washed 3 x with PBS-FCS and resuspended in Eagle s Minimum Essential Medium (monolayer modification) containing 2 mM/ml L glutamine 100 units/ml penicillin 100 µg/ml streptomycin (Grand Island Biological Grand Island N Y) and supplemented with 10 per cent FCS L leucine was excluded from the medium when the H^3 L leucine incorporation was studied

Cell counts were performed using the Natt Herrick stain (5) and the cell suspension was diluted to desired cell concentration with the complete medium

Cotton column filtration The spleen cell suspension was prepared as described above but the PBS contained 50 per cent (v/v) autologous fresh heparinized (20 U/ml) chicken plasma and 1 per cent (w/v) dextran m w 250 000 (Pharmacia Uppsala Sweden) instead of FCS The spleen cell suspension was poured onto a column containing an equal volume of cotton wool (Red Cross Cotton Johnson and Johnson New Brunswick N J) incubated for 15 minutes at 37 C and then eluted with two volumes of the PBS chicken plasma dextran solution

The cell suspension was then centrifuged and the cells were washed 3 x with PBS FCS and finally suspended in complete tissue culture medium

Tissue culture 1 ml volumes of the cell suspension were added to 16 x 100 mm disposable flint glass tissue culture tubes (Bellco Glass Inc Vineland N J) The tubes were maintained with loosely fitted plastic caps at 40 C in a moist 5 per cent CO_2–95 per cent air atmosphere

Phytohaemagglutinin (PHA) Wellcome phytohaemagglutinin (Burroughs Wellcome and Co London England) was reconstituted with 5 ml of PBS per vial and added in indicated volumes to the cultures

Assay procedures

DNA synthesis The incorporation of H^3 methyl thymidine of the cultured cells during a 4 hour period was taken as a measurement of the rate of DNA synthesis of the cultures and was used as a general indicator of the response of cells to PHA when suitable tissue culture conditions were established 1 µc of H^3 methylthymidine specific activity 6 7 c/mM (New England Nuclear Corp Boston Mass) in 0 050 ml of PBS was added per culture tube After 4 hours the tubes were chilled and centrifuged at 270 x g for 10 minutes at 4 C The supernatants were discarded The cell pellets were frozen and thawed once precipitated and extracted twice with cold 5 per cent (w/) trichloro-acetic acid (TCA) and absolute methanol respectively

RNA synthesis The incorporation of H^3 5 uridine during a 15 minute and a 4 hour period was taken as an indicator of the rate of RNA synthesis The short pulse was used in an attempt to measure preferentially rapidly labelled RNA 5 µc H^3 5 uridine specific activity 4 c/mM (Schwarz Bioresearch Orangeburg N Y) in 0 050 ml of PBS was added per culture tube The shorter pulse was

tween 8 and 16 hours PHA caused an increased uptake compared to the control cultures with a maximum at 24 hours Little absolute increase over the incorporation at zero time was noted

Protein synthesis (Fig 9) PHA increased the incorporation of H^3 leucine starting during the first 4 hour pulse. A maximum was noted at 36–40 hours

The DNA RNA and protein synthesis in PHA stimulated cultures decreased at the end of the culture period

The data presented in Fig 6–9 were rearranged to better evaluate changes in cell metabolism during the culture period The mean net increases in isotope incorporation per culture at each pulse interval were calculated by subtracting the incorporated radioactivity (counts per minute) of cultures without PHA from cultures with PHA Such data plotted against time as in Fig 10 may demonstrate the absolute growth rate of the *rate of incorporation of the isotopes* Fig 10C demonstrates that PHA changed the rate of incorporation of H^3 thymidine little until the 16–20 hour pulse but then increased it rapidly until 36–40 hours The incorporation of H^3 uridine determined by 4 hour pulses (Fig 10B) and 15 minute pulses (Fig 10A) showed a linear increase starting within the first hours after the addition of PHA and continuing through the 36–40 hour pulse. A linear increase of the rate of incorporation of H^3 leucine was also noted beginning within the first 4 hours and continuing through the pulse at 36 hours (Fig 10D) The slope of the regression lines indicated in Figure 10 tend to be steeper for the bursectomized irradiated than for the control irradiated group The only statistically significant difference was noted for the incorporation of H^3 uridine using *4 hour pulses* ($t = 2\ 36$, d f 6 0 025 > P > 0 01)

DISCUSSION

Th ptimal conditions for the *in* *o* res ·o ı ı spleen cells to the utogen n (PHA) were tablished Cotton column filtration and a 12 hour incubation of the spleen cells prior to the addition of the PHA yielded highly PHA reactive cells with relatively stable metabolism throughout the culture period. The cotton column filtration possibly removed cell debris and thrombocytes from the spleen cell preparations The latter cells when present in cultures of peripheral chicken blood lymphocytes will inhibit the PHA response (2)

During the 12 hour pre incubation the cell metabolism, represented by the DNA synthesis decreased considerably due to either cell death or conversion of rapidly dividing cells into a resting state The reactivity of the incubated cells to PHA remained intact

The effect of PHA on the metabolism of cultured peripheral blood or thoracic duct lymphocytes is well established (14) The response of the chicken spleen cells to PHA followed the established patterns The RNA and protein synthesis accelerated within 4 *hours and the DNA synthesis increased with* a delay of 16 hours after the addition of PHA While the PHA induced increase of the rate of incorporation of isotopes into RNA and protein showed a linear relationship with time during at least the first 24 hours after the addition of PHA this was probably not true for the increase of the H^3 thymidine into DNA A possible reason for this is that the increase of the RNA and protein synthesis is not immediately associated with cell division but the DNA synthesis is

Spleen cells from normogammaglobulinaemic control irradiated (Cx) and severely hypogammaglobulinaemic bursectomized irradiated (Bx) chickens responded in all but one respect equally well to PHA The exception was the rate of increase of the PHA induced RNA synthesis (4 hour pulses) which *was significantly higher* for the Bx animals. This may indicate that in a given number of spleen lymphoid cells the Bx chickens have proportionally more PHA reactive cells or alternatively that each reactive cell is capable of a more rapid increase of the rate of synthesis of RNA The former explanation appears more likely because the absence of the

immunoglobulin producing cells in hypogammaglobulinaemic chickens (1 6) would tend to increase the proportion of other cell types

The results of this investigation confirm and extend previous findings with respect to the PHA induced DNA synthesis in spleen cells from Bx chickens (1 6) The finding of normal magnitudes and dynamics of the increases of DNA RNA and protein synthesis in such cells rule out any deficiency of the PHA response similar to that described in acquired agammaglobulinaemia in man (4, 12 18) It is well established that surgical bursectomy at hatch coupled with sublethal irradiation will depress in later life antibody formation as well as the serum levels of immunoglobulin and the number of spleen plasma cells (6) The absence of the antibody producing and immunoglobulin containing and producing cells in the spleens of hypogammaglobulinaemic chickens have been demonstrated (1) Apparently the functions of the lymphoid system represented by the antibody response and the *in vitro* response to PHA can be completely dissociated by the procedure of bursectomy and irradiation

It is possible that the response to PHA is a function of thymus dependent lymphoid cells and not of the immunoglobulin producing bursa dependent cells Thus thymectomy of the newly hatched chicken impairs the ability of the lymphoid cells to respond to PHA (11 13 3) but not to produce immunoglobulin (3 5) Furthermore in spite of much work it has not been convincingly demonstrated that PHA responsive cells produce immunoglobulin (10 14)

I wish to thank Dr *Raymond D A Peterson* for generous support during this investigation and Mrs *Clara Maynie* for expert technical assistance

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Assay of antibody forming cells (Table 3) Table 3 shows the number of plaque forming cells (PFCs) per 10^6 spleen cells as well as per spleen in the four experimental groups The results of the use of the direct and in direct versions of the Jerne technique are in dicated separately All means are arithmetic means

The spleens of immunized Cx chickens contained large numbers of both direct and indirect PFCs The mean ratio of the number of indirect PFCs to direct PFCs in this group was 5 20 (range 2 57 to 10 88)

The spleens of six of the immunized Bx chickens lacked detectable PFCs In the re maining two animals small but significant number of PFCs were demonstrated The *findings in these are separately indicated in* Table 3 One of them number 2 of the table appeared to have almost as many direct (132738) as indirect (159407) PFCs

In a significant proportion of the unim munized Cx chickens direct (5/8) and in direct (6/8) PFCs could be demonstrated These PFCs will be referred to as back ground PFCs None of the unimmunized Bx chickens had such background PFCs

DISCUSSION

In the present investigation the antigen in duced increase *in vivo* of the total number of spleen cells was much greater than the number of antibody forming cells (PFCs) de tected by the Jerne technique Others, stu dying the mouse have made similar observa tions (1) Many of these cells which do not form antibody may be thymus—dependent cells because these have in the mouse recent ly been shown to proliferate at antigen chal lenge but not to produce antibod (10–12) *Other proliferating cells may bel* to the same bursa dependent lymphoid c population as the PFCs but produce too e anti body to be detected

The deficient antibody response (ursect om d irradiated (Bx) chickens SRBC cte l m the results of revious

A semiquantitative histological study de monstrated that the development of strongly pyroninophilic, most likely plasmablasts and plasma cells, after antigen challenge of burs ectomized chickens was deficient (16) The finding in the present investigation, of a de ficiency in the antigen induced proliferative spleen response in such animals quantitatively indicate that the thymus dependent lymphoid cells either contribute little to the antigen induced *spleen hyperplasia in the control* animals or are deficient in the proliferative response to antigen in the Bx chickens

If the antigen induced spleen hyperplasia is caused mainly by the proliferation of bursa dependent lymphoid cells, the results suggest that not only the expression of the antibody *forming capacity, but also the proliferation of* these cells is deficient after bursectomy and ir radiation

The lower H^3 thymidine incorporation in the spleens of unimmunized Bx than in the control irradiated (Cx) chickens may be ex plained by the failure of the development of *the immunoglobulin producing cells* and the germinal centres after bursectomy and irradi ation (6, 7) The germinal centres in partic ular are wellknown as foci of intense lymphoid cell proliferation (13 14)

The spleens of unimmunized Cx but not Bx chickens contained significant numb rs of cells forming antibody to SRBC These back ground PFCs of both direct and indirect type, are most likely not the actual precursors of the PFCs in the primary immune response (18, 21) but may develop from these after stimulation by antigens which cross react with SRBC The back ground *PFCs dis* appear in mice made tolerant to SRBC (1) Therefore their absence in the *Bx chickens* indicate that the immunizing dose of SRBC used in the present investigation is *not tolero* genic in the Bx animals

Thymectomy in the mouse impairs the pri mary response to SRBC but does not affect the number of background *PFCs* (2 15) and thus differs in *the latter re* pect from the bursectomy in the chickens

The absence of background PFCs as well

as the proliferative and PFC responses in the spleens of the Bx chickens would support the contention that the precursors for the antibody forming cells in these animals are either absent or less likely not properly activated by antigen to proliferation and differentiation into antibody producers

Two immunized Bx chickens showed small but significant numbers of spleen PFCs The finding that one of the animals had as many direct (IgM) as indirect (IgM + IgG + other immunoglobulin classes) PFCs suggests that the spleen of this animal contained only IgM antibody forming cells This extends to the cellular level the finding that a significant proportion of chickens particularly when bursectomized before hatching responds with predominantly IgM antibody production on antigen challenge in later life (4 5) Bursectomized chickens with or without irradiation have also previously been shown to have decreased blood levels of IgG but sometimes normal or increased levels of IgM (3 7 9 22) These findings have been explained by a sequential bursa dependent ontogenetic development of the ability to synthesize IgM and IgG (4 9)

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DEMONSTRATION OF *FRANCISELLA TULARENSIS* (SYN *PASTEURELLA TULARENSIS*) IN SYLVAN ANIMALS WITH THE AID OF FLUORESCENT ANTIBODIES

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Samples of 279 sylvan animals were investigated for tularaemia both by the FA test as well as by histopathological and/or conventional bacteriological investigations For the FA test we used fluorescein isothiocyanate labelled anti F tularensis globulin from a man who had just recovered from tularaemia In 124 cases where all three methods were used there was complete agreement in 85 cases (68 per cent) In 34 cases (27 per cent) the bacteriological investigations were negative whereas the other two methods gave positive results In 105 suspected cases the FA technique was compared with histopathological procedures only The results were in agreement in 103 cases In 146 guinea pigs which had been inoculated with material from suspected cases the results of cultivation and FA procedures were in agreement in 136 cases (93 per cent) In experimentally infected rabbits tularaemia bacteria could be detected in the liver even after storage at room temperature for 25 days *post mortem* The present results indicate that the FA technique for the detection of tularaemia in field material is a diagnostic method considerable more reliable than inoculation into guinea pigs Furthermore the FA technique is more rapid and eliminates to an essential degree the risk of laboratory infections

The fluorescent antibody technique has been successfully used to demonstrate *F tularensis* in experimentally infected animals (3 5 7 11) Carcasses of naturally infected animals have been investigated in the same manner (4 8 9) with good results The field material investigated however consisted of only a few cases

In connection with a severe epidemic outbreak of tularaemia in the north of Sweden in 1967 (2 10) a large number of sylvan animals from that region were examined for the occurrence of tularaemia We have used this material to test the suitability of the FA method to demonstrate *F tularensis* directly in naturally infected animals

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MATERIAL AND METHODS

The material consisted of 279 dead sylvan animals predominantly hares which were picked up in the fields within the above mentioned epidemic area in the north of Sweden during the spring and early summer of 1967

5 adults rabbits were used in an experimental study of the infection

Bacteriological examination The samples usually liver and spleen from suspected animals were suspended in physiological saline and 2 ml of the suspensions were injected i p in guinea pigs The guinea pigs were kept in separate boxes After 30 days all surviving animals were killed Cultivation was carried out on the solid medium described by

Gaspar et al (6) The diagnosis was considered as positive if the following requirements were fulfilled 1 At autopsy the test animal showed typical gross lesions 2 there was a typical growth on the medium used and 3 the bacteria showed the typical morphology macro- and microscopically Some of the isolated strains were also tested by the agglutination technique

Histopathological examination The differences in the lesions caused by *F tularensis* and other infective agents in hares have recently been discussed by *Borg et al* (1) and we have used the methods and criteria reported by these authors

Preparation of conjugate Serum from a man who had just recovered from tularaemia and presented a high agglutination titre (1 10 000) was precipitated in a semi saturated solution of ammonium sulphate After centrifugation the precipitate was dissolved in a volume of distilled water corresponding to the original volume of the serum Ammonium sulphate was removed from the globulin solution by passage through a column of Sephadex G 25 in 0 01 M phosphate buffer pH 7 8 The absence of ammonium sulphate was controlled by the addition of barium chloride The salt free globulin solution was conjugated with fluorescein isothiocynate (FITC) by the method described by *Cherry et al* (2) The conjugate was then passed through the Sephadex G 25 as described above For further purification the conjugate was diluted and passed through a column packed with DEAE cellulose in 0 02 M phosphate buffer pH 8 0 The conjugate was eluted stepwise with the phosphate buffer containing increasing amounts of sodium chloride (0 03 M 0 05 M 0 14 M and 0 28 M) The main part of the globulin containing fluorescent antibodies was in the fraction obtained by elution with the highest molarity of the buffer This fraction was collected and concentrated by means of dialysis against crystalline sucrose to double the original volume of the serum

The conjugate was titrated with a non virulent vaccine strain and the highest dilution that produced a strong fluorescense with brilliant margins of the bacterial cell was taken as the FA titre of the conjugate For most of the conjugates this value was 1 80 Such conjugates were used in a working dilution of 1 10

Preparation and Staining of Imprint Smears

Cut surfaces of livers and spleens were imprinted on microscope slides After the preparations had been air dried and heat fixed they were treated with a drop of the conjugated anti F tularensis globu n with the addition of counterstai conju te t incub tion in a moist chamber t 37 C the preparations w e rinsed minutes in 0 0 M phosphate buffer pH 7 8 and finally mounted with a cover glass and glycerine buffer pH 7 8

Counterstaining Technique with Lissamine Rhodamine Conjugated Bovine Albumin (Difco)

FITC conjugated anti F tularensis globulin in double working concentration was mixed with an equal volume of lissamine rhodamine conjugated bovine albumin in a dilution of 1 10 and this mixture was used for staining the imprint smears

Fluorescense microscopy A Zeiss fluorescense microscope with a mercury lamp Osram HBO 200 and excitation filter B G 12 and secondary filters 53/44 was used A magnification of 600 × was mostly used

Evaluation of FA Stained Imprints Controls

One smear from each organ was stained as described above The criteria of a positive FA test was the repeated occurrence of fluorescent bacteria of similar morphology in different fields and no fluorescent bacteria were found in the two control smears The control smears were tested by staining with a) FITC conjugated globulin from a non immunized rabbit and b) anti F tularensis conjugate in double working concentration mixed with an equal volume of undiluted unconjugated anti F tularensis serum (one step inhibition test)

Experimental Infection

Three adult rabbits were given lethal doses of *F tularensis* subcutaneously and after death imprint smears were taken from spleen and liver every 2–3 days The organs were left *in situ* at room temperature for one week and were then stored in petri dishes at room temperature For control tests two healthy rabbits were killed at the same time and the corresponding organs from these animals were treated in the same way

RESULTS

In experimentally infected rabbits *F tularensis* could be detected in the liver even after storage at room temperature for 25 days *post mortem* The number of fluorescent bacteria and the intensity of their fluorescence however gradually decreased from about the 10th day *post mortem* In the spleen the bacteria were autolysed from about 7th day *post mortem* and from this time we observed fluorescent granules and fragments of the bacteria with gradually weakening fluorescence

In a field material the FA technique was

TABLE 1 *Comparison of the Direct FA Technique Histopathological examination and Inoculation of Guinea Pig* for the Diagnosis of Tularaemia*

FA technique Autopsy Inoculation	+ + +	+ + −	+ − +	− + +	+ − −	− − +	− + −	− − −	Total
Hares	30	33	0	1	3	0	0	3[illegible]	104
Various wild animals	0	0	0	0	1	0	0	10	11
Domestic animals	0	1	0	0	0	0	0	8	9
Total	30	34	0	1	4	0	0	55	1[illegible]4

* Confirmed by cultivation procedures

compared to histopathological and/or conventional bacteriological findings As seen in Table 1 complete agreefent was obtained in 85 cases (68 per cent 30 positive 55 negative) In 34 cases (27 per cent) the results of the histopathological and FA investigations were positive whereas the bacteriological examinations were negative 4 cases were positive with the FA technique but could not be confirmed with the other methods In one single case the FA technique gave a negative result whereas the other two diagnostic procedures were positive

In a material consisting of 105 suspected cases the FA technique was compared with histopathological procedures only The results (Table 2) were in agreement in 103 cases (98 per cent 57 positive 46 negative) The remaining 2 cases were positive histopathologically but negative with the FA technique

TABLE 2 *Comparison of the Direct FA technique and Histopathological Examination for the diagnosis of Tularaemia*

FA technique Autopsy	+ +	+ 	− +	− −	Total
Hares	57	0	2	[illegible]6	85
Various wild animals	0	0	0	1[illegible]	12
Domestic animals	0	0	0	8	8
Total	57	0	[illegible]	46	105

In 146 guinea pigs used for the bacteriological investigations and which had been inoculated with material from suspected case of tularaemia infection the results of cultivation and FA procedures were compared (Table 3) Both methods gave the same diagnosis in 136 cases (93 per cent In 9 cases (6 per cent) the FA technique gave a positive result whereas the cultivation was negative In one single case the FA test was negative whereas the cultivation method was positive.

DISCUSSION

The results of this study on the reliability of the FA test for the detection of tularaemia in dead animals are in good agreement with the results reported by *Franek & Wolfova* (4) and *Mc Cahan et al* (3) The first mentioned authors found full agreement between positive FA test of tissue smears and positive biological

TABLE 3 *Comparison of the Direct FA technique and Cultivation Procedures for the Diagnosis of Tularaemia*

FA technique Cultivation	+ +	 −	+ −	− +	Total
Guinea pig	52	84			[illegible]

Gaspar et al (6) The diagnosis was considered as positive if the following requirements were fulfilled 1 At autopsy the test animal showed typical gross lesions 2 there was a typical growth on the medium used and 3 the bacteria showed the typical morphology macro- and microscopically Some of the isolated strains were also tested by the agglutination technique

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* *Confirmed by cultivation procedures*

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FA technique Cultivation	+ +	− −	+ −	− +	Total
Guinea pig	52	84			

test in an investigation of 4 dead hares *Mc Cahan et al* arrived at the same agreement between FA test and cultivation in a similar examination of 3 dead hares

Despite the use of the same working dilution of the conjugate, there was a marked difference in the intensity of the fluorescence between bacteria from different cases apparently due to varying degrees of decomposition Since *F tularensis* is a small organism the presence of fluorescent granula makes the diagnosis difficult Therefore it is important to ascertain that the morphology of fluorescent particles agrees with that of the organism studied In the fluorescense microscope *F tularensis* has the appearance of small cocci of which only some have clearly defined fluorescent contours and a dark centre In many cells the fluorescence of the central part is of almost the same intensity as that of the periphery Sometimes the pasteurella appeared in aggregates

As pointed out earlier by *Franek & Prochazka* (5) a reliable diagnosis of tularaemic infection with FA technique cannot be made on the basis of findings of single pasteurella like bacterium in the imprint smears The result can be regarded as definitely positive only if the smear contains at least a few fluorescent bacteria of the typical morphology

As has been shown by *Borg et al* (1) histopathological examination is a very reliable method of establishing the diagnosis of tularaemia in hares and the results obtained with the immunofluorescent technique are in good agreement with the results obtained with this method

On the other hand inoculation in guinea pigs failed to demonstrate tularaemia in about 30 per cent of the cases found to be positive with the other two diagnostic procedures In a field material with a high degree of decomposition of course the contaminating bacterial flora makes the bacteriological examination very difficult and in several cases it is doubtful whether the causative agent is still viable

In a few cases there was a discrepancy between the results of the histopathological technique and FA technique (Tables 1 and 2) When the former procedure was positive and the FA technique negative (3 cases of 229) this might be explained by the fact that the bacteria were autolysed in too high a degree or that the bacteria were unevenly distributed in the organs In the guinea pig material (Table 3) where 9 cases (6 per cent) were positive by FA technique but negative by isolation at least 5 animals showed gross lesions that indicated tularaemia

Our investigations on experementally infected rabbits indicated that the FA technique for the detection of tularaemia also appears to give reliable results in highly decomposed material (in our experiments the material was serviceable for up to 25 days at room temperature) This observation should have relevance in the handling of samples collected from animal field cases

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BRIEF REPORTS

EFFECTS OF PARTIAL HEPATECTOMY ON THE IMMUNE RESPONSE TO SHEEP RED CELLS

Preliminary report

Akiyoshi Sakai C N Muller Berat M Debray Sachs and M C Creton*

Little is known about the control of antibody synthesis in higher organisms The central role of the liver in protein metabolism and the fact that a major resection of the liver not only leads to increased DNA synthesis in the regenerating liver cells (1) but also in lymphoid tissues as measured by ^{3}H thymidine uptake (2) indicates that the liver may play some role in modifying the antibody response to any given antigen A series of experiments has been carried out to explore the effect of partial hepatectomy on antibody production in the rat following immunization with sheep red blood cells (SRC)

MATERIAL AND METHODS

Female Wistar rats 100–150 g body weight were immunized with a single intravenous injection of 2 5 x 10[9] SRC Partial hepatectomy was carried out at various time intervals before and after immunization Removal of left and median lobes using *Higgins & Anderson* s method (3) is referred to as) per cent hepatectomy The animals were then bled serially Serum was stored at –30 C until com ion of each experiment Hemolysin and hemagglutinin titres were measured by the microtitration technique at varying time intervals after immunization and partial hepatectomy Double dilution of the sera was made with 1 per cent inactivated normal rat serum in physiological saline and mixed with 0 5 per cent three times washed SRC suspension The readings were made after standing overnight at room temperature Hemolytic plaque forming cells (PFC) were studied with pleen cell suspensions according to *Jerne* s techniq e (4) using lyophilized guinea pig compleme obtained from the Institut Pasteur (Paris) and rosette forming cells (RFC) according to the method of *Bio* (5) For the study of DNA kinetics after partial hepatectomy ^{3}H thymidine 0 5 μg/g body weight was given intravenously and ^{3}H DNA specific activity was counted in cell suspensions prepared from spleen thymus and lymph nodes *In vitro* incorporation of ^{14}C thymidine into lymphoid cell preparations was also studied at varying time intervals before and after immunization and partial hepatectomy The cell suspensions (4 ml with 7500 cells/mm^{3}) were incubated for 4 hours with 0 6 μCi of ^{14}C thymidine The counting was made with a Packard well type scintillation counter The viability of these cell preparations as measured with the trypan blue exclusion test varied from 75 to 94 per cent

Results

When partial hepatectomy was performed *after* immunization with SRC the number of PFC and RFC was markedly increased A representative experiment is shown in Fig 1 where hepatectomy has been performed 2 days after immunization and PFC studied serially The peak response is seen at day 4 as in the control groups This is followed by a sharp decrease of PFC suggesting the effect of hepatectomy to be transitory Similar re ults with the same sequence were observed with RFC (Fig 2) On the other hand serum hemolysin and hemagglutinin titres were not significantly modified after hepatectomy

Accordingly we investigated the effect of partial hepatectomy performed serially at daily intervals after immunization on the number of PFC RFC and hemagglutinin level as determined 24 hours after hepatectomy (Fig 3) A more dramatic increase of PFC and RFC was observed here than in the afore mentioned experiment the number of PFC and RFC in hepatectomized rats at day 4 was re pectively 10 times and 3 times higher than in control or sham-operated animals Moreover

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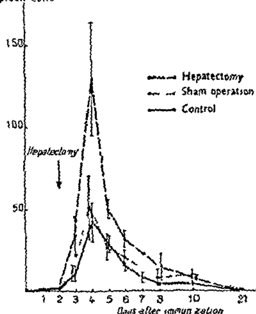


Fig 1 PFC response in Wistar rat spleen after priming with 2 5 × 10⁹ SRC All points represent the results found in groups of five to eight rats (mean ± SD of PFC detected per 10⁶ spleen cells) In this series 70 per cent hepatectomy was carried out 2 days after immunization and assays were performed on each successive day

partial hepatectomy seemed able to shorten the induction period of antibody synthesis as shown by the very early appearance of an increased number of PFC in the spleen of partially hepatectomized rats However when partial hepatectomy was done 10 to 45 days after immunization no rise of PFC or RFC was obtained The titres of hemolysins and hemagglutinins were somewhat increased in hepatectomized rats over sham operated or control animals but the difference was not striking

In vitro incorporation of ^{3}H thymidine into various lymphoid cell suspensions has shown the uptake of DNA precursors to be markedly decreased in thymocytes (135 ± 15 cpm per mg DNA) and increased in splenic cells (3970 ± 757 cpm per mg DNA) 4 days after immunization and one day after hepatectomy when compared with the results obtained in non immunized controls which were 1160 ± 147 cpm per mg DNA and 1360 ± 297 cpm per mg DNA respectively

Partial hepatectomy done 1 2 and 7 days prior to immunization and assayed 1 4 and 6 days after immunization seemed to have no effect on the immune response It was also examined if partial hepatectomy per se could induce an increase of naturally occurring antibody producing cells in non immunized rats Neither the level of circulating antibodies nor the number of PFC and RFC were significantly increased on days 1 4 and 7 after operation although *in vivo* ^{3}H thymidine uptake in hepatectomized animals vs controls or sham operated rats was markedly increased 3 days after partial hepatectomy either in the spleen (3020 ± 1080 cpm per mg DNA vs 1460 ± 360 cpm) thymus (1650 ± 350 cpm per mg DNA vs 270 ± 90 cpm) or lymph nodes (2260 ± 560 cpm per mg DNA vs 1060 ± 210 cpm)

Discussion

Our results indicate that partial hepatectomy in the rat increases the magnitude of the primary antibody response to sheep erythrocytes as measured by the number of PFC and RFC in the spleen This happens provided hepatectomy is performed after the antigenic stimulus i e when antibody forming cells (AFC) are already triggered into proliferation and differentiation upon contact with the antigen The peak of response is not modified in its timing No apparent increase of serum antibody could be found

This augmentation of the number of AFC and RFC could be accounted for either by a shortening

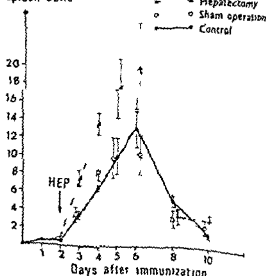


Fig 2 RFC response in Wistar rat spleen after priming with 1 2 × 10⁹ SRC. All points represent the results found in groups of five to eight rats (mean ± SD of RFC detected per 10³ cells) In this series 70 per cent hepatectomy was carried out 2 days after immunization and assays were performed on each

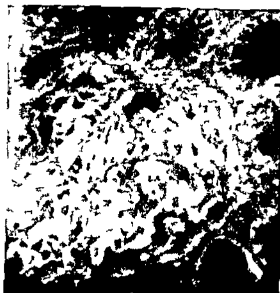


Fig 1 Renal biopsy treated with anti beta 1 C conjugate showing deposition in the glomerulus and along some of the tubular basal membranes



Fig 2 Renal tissue treated with anti beta 1 C conjugate Note staining of the thick wall of an arteriole

parts of the glomerular capillary loops other glomeruli of *the same biopsy either showed* a fine threadlike fluorescence of the basement membrane or were completely negative In the biopsy from the patient with the nephrotic syndrome the glomeruli all showed a strong linear fluorescence of the glomerular capillary basement membrane and a more uneven staining of the mesangial tissue (Figs 1 and 2) The staining for the three specific immunoglobulins as well as for whole gammaglobulin was remarkably faint as compared with the intense staining of the corresponding section stained for complement A slight staining for IgM was seen as a thin lining in the glomerular capillary loops in the nephrotic patient, whereas other immunoglobulins could not be found either in the glomeruli or in the walls of the small arteries or arterioles Staining for albumin and fibrinogen was negative and no useful informations were obtained with the other conjugates

Discussion

In patients with the acute polyradiculitis syndrome the main interest in autopsy examinations has naturally been concerned with ce ral and peripheral nerve tissues and the literat has made little mention of any changes of the els However our own descriptions of autop ections of nerve tissues obtained in previous se did in a ses point out a remarkable thic ing of the vessel walls as well as infiltration with inflammatory mononuclear cells The e changes may partly correspond to tho e of the renal vessels here reported If similar changes in vessels from other organs al o could be proven they might possibly explain some of the described cardiac and vascular disturbances in patients with this syndrome (1) The papilloedema occasionally observed (2) might have a similar vascular pathogene is

The alterations described suggest the presence of soluble circulating antigen antibody complexes as is the case in patients with auto immune renal diseases The unexpected findings of the same deposits *especially of complement even in the present* series of five patients with no overt renal involvement accord with our experience of renal biopsies from some patients with chronic hepatitis (4) also from those with normal renal function Thus such changes may be common phenomena in a variety of diseases in which immunological mechanisms are involved The dissociation of complement and immunoglobulins observed in the present series has also been observed in some patients with systemic lupus erythematosus and with other diseases It may be due to an involvement of other immunoglobulins or to a mobilization of complement by some pathogenetic factor without any activation of immunoglobulins

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AUSTRALIA ANTIGEN IN PATIENTS WITH VARIOUS LIVER DISEASES

PREBEN ELLING, JENS O NIELSEN and ODD DIETRICHSON

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The Pathological Anatomical Institute Copenhagen Municipal Hospital
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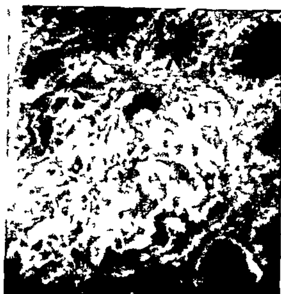


Fig 1 Renal biopsy treated with anti beta 1 C conjugate showing depo ition in the glomerulus and along some of the tubular basal membranes



Fig 2 Renal tissue treated with anti beta 1 C conjugate Note staining of the thick wall of an arteriole

parts of the glomerular capillary loops other glomeruli of the same biopsy either showed a fine threadlike fluorescence of the basement membrane or were completely negative In the biopsy from the patient with the nephrotic syndrome the glomeruli all showed a strong linear fluorescence of the glomerular capillary basement membrane and a more uneven staining of the mesangial tissue (Figs 1 and 2) The staining for the three specific immunoglobulins as well as for whole gammaglobulin was remarkably faint as compared with the intense staining of the corresponding section stained for complement A slight staining for IgM was een as a thin lining in the glomerular capillary loops in the nephrotic patient whereas other immunoglobulins could not be found either in the glomeruli or in the walls of the small arteries or arterioles Staining for albumin and fibrinogen was negative and no useful informations were obtained with the other conjugates

Discussion

In patients with the acute polyradiculitis syndrome the main interest in autopsy examinations has naturally been concerned with cet ral and peripheral nerve tissues and the literat has made little mention of any changes of the sels However our own descriptions of autop ections of nerve tissues obtained in previous se did in a es point out a remarkable thicl ng of the vessel walls as well as infiltration with inflammatory mononuclear cells The e changes may partly correspond to those of the renal vessels here reported If similar changes in vessels from other organs also could be proven they might pos ibly explain some of the described cardiac and vascular disturbances in patients with this syndrome (1) The papilloedema occasionally observed (2) might have a similar vascular pathogenesis

The alterations described suggest the presence of soluble circulating antigen antibody complexes as is the case in patients with auto immune renal diseases The unexpected findings of the same deposits especially of complement even in the present series of five patients with no overt renal involvement accord with our experience of renal biopsies from some patients with chronic hepatitis (4) also from those with normal renal function Thus such changes may be common phenomena in a variety of diseases in which immunological mechanisms are involved The dissociation of complement and immunoglobulins observed in the present series has also been ob erved in some patients with systemic lupus erythematosus and with other di eases It may be due to an involvement of other immunoglobulins or to a mobilization of complement by some pathogenetic factor without any activation of immunoglobulins

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Acta path microbiol scand Section B 78, 657–663 1970

AUSTRALIA ANTIGEN IN PATIENTS WITH VARIOUS LIVER DISEASES

PREBEN ELLING JENS O NIELSEN and ODD DIETRICHSON

The Department for Infectious Diseases Blegdams Hospital Heads V Faber and P Efferso
Medical Department 2 Copenhagen Municipal Hospital Heads Kurt Iversen and A Skouby
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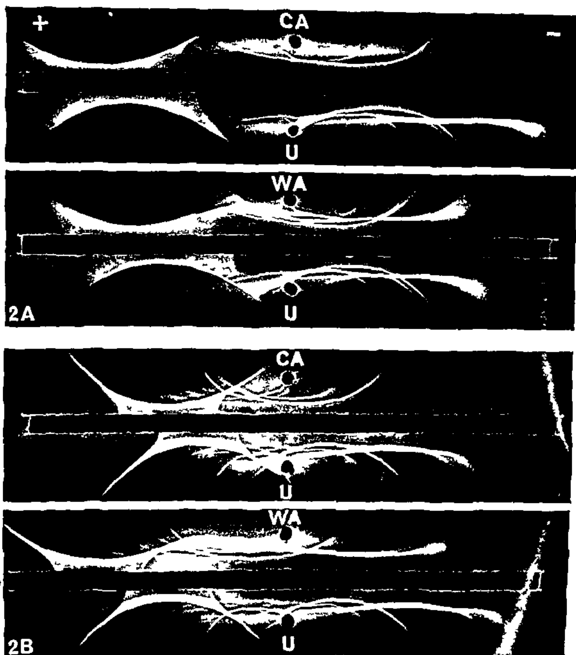


Fig 2 Immunoelectrophoretic analysis of canine (A) and porcine (B) sera before (U) and after two absorptions with *S aureus* Cowan type I (CA) or *S aureus* Wood 46 (WA) Unabsorbed serum (U) was used as control on each slide All samples were tested with homologous antisera Note that several components in the absorbed samples had a greater mobility than the corresponding components in unabsorbed samples.

formed by the NHS Between protein A and sera from cow and sheep one or two weak lines were seen

The antigen prepared from strain Wood 46 according to the method of *Jensen* 10) did not precipitate any of the sera exai ned

Absorption of Sera

Effect upon reactivity in the precipitation reaction The changes observed by means of the microtechnique were the same as those shown for conjugates in Fig 1 After one absorption with *S aur us* Cowan type *I* less

marked precipitates were formed between protein A and porcine and canine sera while no reactivity remained with NHS After two absorptions no precipitate was visible between protein A and canine serum while the porcine serum still yielded a slight degree of reaction In the sample of NHS absorbed twice with Cowan I a surplus of protein A present reacted with the samples in the adjacent wells (see Fig 1 C)

By the more sensitive macrotechnique we obtained similar results *i e* the precipitation lines gradually disappeared after absorptions with Cowan I Only the porcine serum retained a weak reactivity after two absorptions

None of the absorptions with Wood 46 had any effect on reactivity in the precipitation reaction

Qualitative and quantitative changes in protein content

Immunoelectrophoretic analysis Among the immunoglobulins which were demonstrable by immunoelectrophoretic analysis of the sera were the following γG globulins the antigenically related fast and slow γG globulins clearly distinguishable in bovine ovine and canine sera by their homologous antisera (Figs 3 and 2 A) the fast γG globulin of porcine serum only seen as a rather faint spur of slightly greater mobility than that of slow γG globulin and the human γG globulin always seen as one continuous precipitation line.

A comparison of unabsorbed and absorbed sera by immunoelectrophoretic analysis and agar gel electrophoresis served to evaluate the qualitative and quantitative changes of serum proteins

The human, canine and porcine sera were almost completely depleted of γG globulins after two absorptions with *S aureus* Cowan type I By agar gel electrophoresis no protein was demonstrated in the γ mobility range and by immunoelectrophoretic analysis only trace amounts of γG-components were found (Fig 2) The remaining quantities were estimated to be less than 1/32 of the original amounts

The slow γG components of bovine and ovine sera were also completely removed after two absorptions with Cowan I, whereas the fast γG components of these two species were left unchanged (Fig 3) The changes found for bovine serum were confirmed by the use of the non anti slow γG globulin and non anti fast γG globulin sera (see *Materials and Methods*) (Fig 4)

A slight decrease in concentration of other serum proteins and especially of immunoglobulins was seen in sera from the five species investigated These changes were due to the unavoidable dilution of the samples by absorption A similar dilution effect was found by investigation of the serum samples absorbed with Wood 46 *i e* the γG components which were removed by the reactive strain were only slightly affected by the absorption with Wood 46 (Figs 2 & 3)

Absorption of Conjugates

Effect on staining of S aureus in FAT Absorptions with *S aureus* Cowan type I re

TABLE 2 *Non-Specific Immunofluorescent Staining of* S aureus *Cowan Type I by Various Conjugates before and after Absorptions with* S aureus *Cowan Type I and* S aureus *Wood 46*

FITC labelled globulin from	Degree of fluorescence at dilution 1 40 of conjugate				
	before absorption	after absorption with S aureus			
		Cowan I		Wood 46	
		x 1	x 2	x 1	x 2
Man	+ + + +	+ +	0	+ + + +	+ + + +
Dog	+ + + +	+ + + +	0	+ + + +	+ + + +
Swine	+ + + +	+ + + +	+	+ + + +	+ + + +
Cow	+ + +	0	0	+ + +	+ + +
Sheep*	+ + +	0	0	+ +	+ +

* Tested undiluted

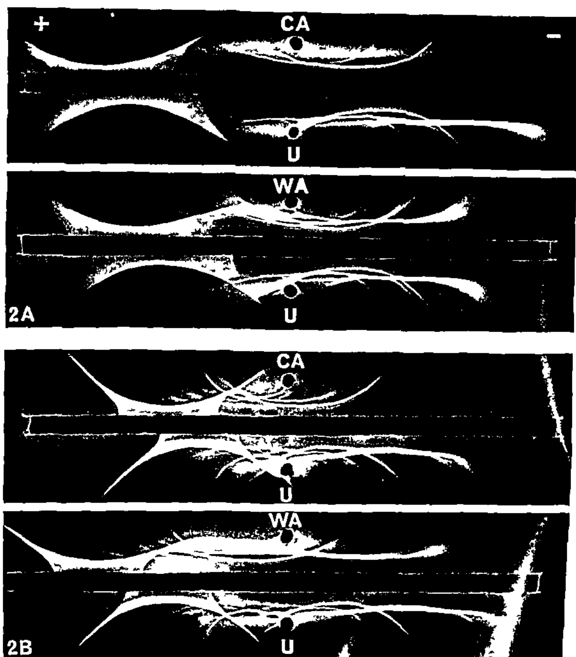


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Swine	+ + + +	+ + + +	+	+ + + +	+ + + +
Cow	+ + +	0	0	+ + +	+ + +
*Sheep**	+ + +	0	0	+ +	+ +

* Tested undiluted

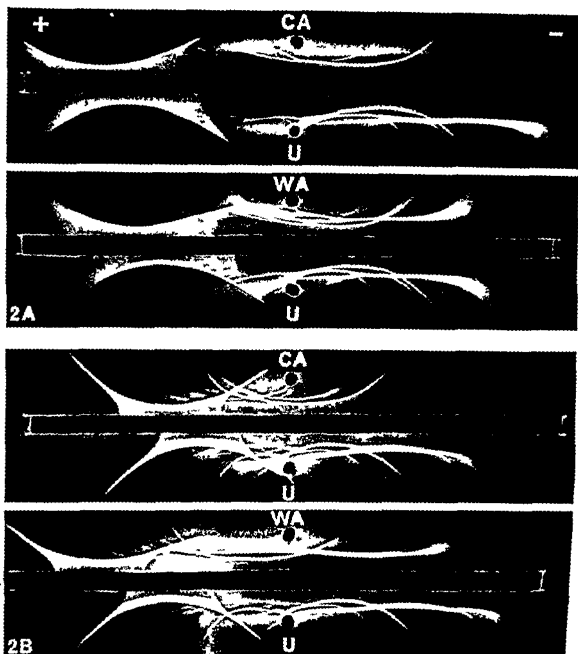


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UTILIZATION OF THYMINE, THYMIDINE AND TMP BY NEISSERIA MENINGITIDIS

1 Growth Response and Uptake of Labelled Material

SIDSEL JYSSUM and KAARE JYSSUM

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
Rikshospitalet Oslo Norway

Attempts to isolate thymine thymidine or TMP deficient mutants from *Neisseria meningitidis* were altogether negative This led to the examination of the growth response and the uptake of these compounds when exogenously added The growth rate was slightly increased upon their addition to cultures of meningococci adapted to growth on basal media At the same time radioactivity was taken up from labelled thymine to an extent of 5 6 per cent of the material added from thymidine to an extent of 0 6 per cent and from TMP to an extent of 0 05 per cent The addition of purine deoxyribonucleosides or the base analogue 5 fluorodeoxyuridine did not increase the uptake of labelled material from thymidine. Growth inhibition which was observed upon the addition of deoxyadenosine 5 fluorodeoxyuridine or 5 fluorouracil has been discussed

The incorporation of ^{3}H and ^{14}C labelled thymine or thymidine has often been used as a simple and reliable technique for the labelling of newly synthesized DNA In many bacterial species thymidine can be used for specific labelling of DNA in prototrophs as well as in auxotrophs

In *Escherichia coli* prototrophs thymine or thymidine is incorporated specifically into DNA when a deoxyribonucleoside is added to the culture medium due to the enzymes nucleoside deoxyribosyltransferase and thymidine kinase (6 29) *Rachmeler et al* (30) described the breakdown of thymidine by *E coli* and Budman and Pardee (9) later reported that the addition of other nucleosides inhibited the break down of thymidine allowing more of it to be taken up *Breitman et al* (7) showed that wild type *E coli* grown in the presence of labelled exogenous deoxythymidylic acid (TMP) incorporate label into DNA at a constant rate for extended periods of time But exogenously supplied pyrimidine nucleotides are not incorporated into nucleic acids *in toto* They are rather dephosphorylated before utilization of the pyrimidine moiety (10 26)

Bodmer & Grether (5) found low efficiency of incorporation of thymine compared with that of thymidine in *Bacillus subtilis* results which parallel the results reported by other workers with *E coli* and other microbes (8 13 34) Thymidine is incorporated into *B subtilis* DNA to an extent c 99 per cent whereas from 1 to 2 per cent in RNA (5)

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Addendum June 9 1970

Owing to the postal strike in U S A the January issue of J Immunol did not arrive at our library before the end of May i e after the present article had been submitted for publication In the J Immunol *104* 140–147 1970 *Kronvall et al* publish studies on the reaction between protein A of staphylococci and sera or isolated γG globulins from species representing even classes and 30 orders of living vertebrates They used a precipitation method or inhibition of precipitation for testing protein A reactivity Their study included the five species investigated by us The results seem to be in agreement since their finding that sera of ruminants gave a weak reaction (a ± inhibition of precipitation) is compatible with our finding namely that only the slow γG globulin component of cow and sheep sera exhibited reactivity with protein A of *S aureus*

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TABLE 4 *Incorporation of ³H from Labelled Thymidine in* Neisseria meningitidis *Strain MI*

Expt No	Supplements added to basal medium			Incorporation of ³H in terms of μg thymidine per viable colony forming unit
	³H thymidine	Other chemicals		
1	1 μg/ml	None		$3\,2 \times 10^{12}$
		Deoxyadenosine	500 μg/ml	$4\,1 \times 10^{-12}$
		Deoxyguanosine		$5\,1 \times 10^{12}$
		Deoxyinosine		$3\,9 \times 10^{12}$
		5 FUdR	50	$4\,7 \times 10^{-1}$
	4	Deoxyinosine	500	$1\,5 \times 10^{11}$
2	1	None		$8\,5 \times 10^{-12}$
		Deoxyadenosine	500 μg/ml	$6\,2 \times 10^{-12}$
		Deoxyguanosine		$5\,? \times 10^{12}$
		Deoxyinosine		$6\,6 \times 10^{-12}$
		5 FUdR	50	$4\,5 \times 10^{12}$
	4	Deoxyinosine	500	$2\,0 \times 10^{11}$

Conditions as described for Table 3 The ³H thymidine plus deoxyribosides were added when the cultures had reached the exponential growth phase

Exogenous TMP is obviously utilized to only a very small extent by growing *N meningitidis* cells (Fig 4) When the uptake obtained with the concentration 1 34 μg/ml is compared with that found with 6 7 μg/ml both concentrations result in an uptake which corresponds to about 0 05 per cent of the drug added This means that TMP must be a very uneconomical source for the labelling of *N meningitidis*

DISCUSSION

The attempts to isolate thymine thymidine or TMP requiring mutants from *N meningitidis* have been altogether negative although control experiments with *E coli* in similar technique were successful This negative finding combined with the isolation of everal mutants requiring adenine plus ianine for growth a finding which is not ical for *E coli* when subject to selective ssure by means of folic acid analogues point to some fundamental difference t een the metabolism of nucleic acid pi irsors in *N meningitidis* and *E coli* On vpothesis is that *N meningitidis* lacks the e ymes mediating the incorporation of exo nous thymine or thymidine into nucleic acids. In contrast, adenine and guanine which are known to be taken up in *N meningitidis* in large quantities (25) may presumably be incorporated This would explain the preferential isolation of mutants requiring adenine plus guanine when employing folic acid analogues since at least two of the reactions involved in the biosynthesis of IMP, the common precursor of AMP and GMP, require folic acid coenzymes.

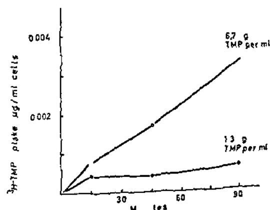


Fig 4 Utilization of exogenous ³H TMP by *N meningitidis* Strain MI Conditions of the experiment as in Table 4

The addition of thymine thymidine and TMP to *N meningitidis* adapted to growth on basal media resulted in a slightly increased growth rate. This effect may very well correspond to an uptake followed by metabolization The findings by *Kingsbury* (25) that *N meningitidis* utilizes exogenous thymine to an extent of 5 6 per cent of added label was confirmed As for thymidine the utilization was maximum 0 6 per cent which is also close to the findings of *Kingsbury* (25) of 0 5 per cent TMP is utilized to an extent of 0 05 per cent only The uptake of these compounds and particularly of thymidine and TMP is thus very limited A calculation which allows for the decomposition (3) indicates that the radioactivity assimilated into the cells from TMP could be due to break down products

Unlike the findings from *E coli* and *Haemophilus* the uptake in *N meningitidis* of thymine or thymidine could not be increased by the addition of deoxyribonucleosides This may also indicate some fundamental difference between *N meningitidis* and the other microbes mentioned The finding that addition of base analogues particularly 5 fluorodeoxyuridine does not increase the uptake of radioactivity from thymine or thymidine may further emphasize that the situation in *N meningitidis* is different from that in *Haemophilus* (32)

The findings from *N meningitidis* may point to a situation similar to that found in organisms like *Neurospora crassa Aspergillus niger Saccharomyces cerevisiae* and *Euglena gracilis* (18) DNA is not specifically labelled in *N crassa* grown in the presence of ^{14}C thymidine ^{14}C TMP or ^{14}C TTP Instead the isotope is incorporated into both types of nucleic acid in a ratio which approximate the ratio RNA to DNA It has been considered that the absence of thymidine kinase prevents the specific incorporation of thymidine into DNA in intact cells of *N crassa* The incorporation of thymidine actually observed is thought to involve the removal of the 5 methyl group leaving a uridine derivative which is then further metabolized to yield the required pyrimidine nucleotides (1 16)

The addition of deoxyadenosine shows a clear inhibition of the growth rate in *N meningitidis* Such an effect has also been reported for certain strains of *E coli* (17) Deoxyadenosine in concentrations of 0 5 M (125 ug/ml) also strongly inhibits growth in Chang liver cells (14) and other mammalian cells (28)

The growth of *N meningitidis* is strongly inhibited by low concentrations of 5 fluorouracil an effect which is reversed by uracil and not by thymine or thymidine In most systems studied both uracil and thymine are needed to overcome the inhibiting effect of 5 fluorouracil (33) But the behaviour of *N meningitidis* is similar to that of a thymidine kinase deficient mutant of *E coli* K12 which has also been shown to be inhibited by 5 fluorouracil even in the presence of uridine and thymidine in the medium (19)

Since the growth inhibition of 5 fluorouracil is abolished by uracil and not by uridine it may be assumed that uridine phosphorylase is absent from *N meningitidis* and that also uridine kinase is absent so that no UMP is formed Therefore a UMP pyrophosphorylase may be the enzyme active in the incorporation of uracil (15)

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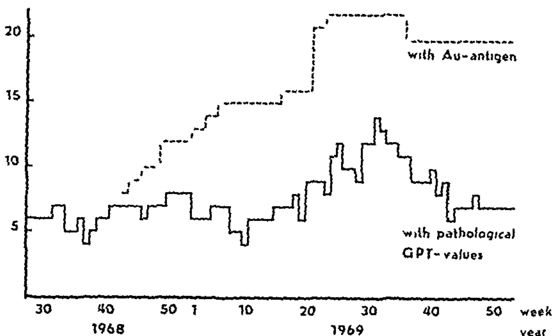


Fig 1 Occurrence of pathological GPT values and Au antigen in 32 hemodialysis patients



The correlation between hepatitis and Australia (Au) antigen is now well established (*Blumberg et al* 1968 *Okochi* & *Murakami* 1968 *Nordenfelt* & *Kjellen* 1969 and others) The SH antigen described by *Prince* (1968) and the hepatitis associated antigen described by *Gocke* & *Kavey* (1969) may now be regarded as identical with Au antigen is connected with ... pes of hepatitis i e the epidemic type with short incubation period and the serum type with a long incubation period or only the last one has been debated Recent evidence speaks strongly for it being associated only with the long incubation type (*Hargrove et al* 1969 *Giles et al* 1969)

Epidemics of hepatitis have occurred at several of the large hemodialysis centers established for treatment of chronic renal disease with hemodialysis (*Forrest et al* ...

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TABLE 1

Comparison of some clinical data between patients and members of the staff	Patients undergoing hemodialysis	Members of the staff	P
Total number with hepatitis	30	30	–
Number with jaundice	4	23	<0 001
Maximal GPT in units mean±S E	383 ±57	1694 ±195	<0 001
Duration of GPT rise in weeks mean±S E	16 0± 2 8 (n=24)	7 7± 0 8 (n=26)	<0 005

From January 1968 all patients receiving hemo dialysis were given 10 ml 16 per cent gammaglob ulin a month When the hepatitis epidemic started those members of the staff who had not had hepa titis were likewise given 10 ml 16 per cent gamma globulin a month After the injections several of the members developed side effects now subjected to a special examination

RESULTS

Patients

The patients receiving regular hemodialy sis treatment were very little affected by their hepatitis Weakness and fatigue which were the commonest symptoms were reported by 9 of the 30 with clinical hepatitis Nausea was reported by 6 while urticaria and joint pain were reported by only a few Only 4 of the 30 had jaundice which was never more than moderate and the highest concentration of serum bilirubin in this group was 4 3 mg/ 100 ml The increase of the GPT was mo derate and the maximal values were on the average 383 ± 57 units (Table 1)

In 25 of the 30 patients with hepatitis Au antigen was demonstrated in the serum The patients could be grouped according to [illegible] ver pi [illegible] ues abo e 1 2 mg per 100 ml were regarded as abnormal

Treatment with hemodialysis was given with a commercially available disposable plate kidney model Alwall (*Alwall* 1963 *Lindholm & Alwall* 1969) As a rule the patients were treated twice a week for 7–8 hours each time and the average consumption of blood per patient was about 400 ml packed red blood cells per month

Definition of hepatitis Hepatitis was said to exist when the GPT was increased and when bilirubinemia was diagnosed and in anicteric cases when the GPT was more than 40 units on at least 2 consecutive samples obtained at least 1 week apart

of the patients in the group have since lost this antigen (Dec 1969)

One group consisted of 7 patients Au antigen was demonstrated in all of them at

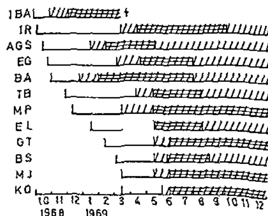


Fig 3 Typical pattern of occurrence of Au antigen in serum in dialysis patients with hepatitis Unbroken lines denotes period covered by samples. This period is identical with that of dialysis in all patients except one (IBA) treated from July 1967
/// denotes demonstration of Au antigen
[illegible]

with a peak in July 1969 when it was increased in as many as 14 patients

Between July 1968 and December 1969 thirty members of the staff had hepatitis The frequency reached a peak in September 1968 (*Fig 2*) after which it decreased and in April 1969 there was no case of hepatitis among the staff It is clear from Fig 1 that the frequency of hepatitis among the patients receiving dialysis was roughly the same throughout this period During the first weeks in July 1969 however two members of staff developed hepatitis and in the au[illegible] 1969 there were always 3–5 members of the staff ill with hepatitis This second outbreak of [illegible] among the staff began at roughly the [illegible] of the year as the first one

dialysis fell ill with clinically clear hepatitis with jaundice From 3 of these, samples were obtained for examination for Au antigen and 2 proved positive

One male relative of a female member of the staff also fell ill with hepatitis with jaundice He had Au antigen at the time of onset In this woman (E B Fig 7) no Au antigen could be demonstrated in samples obtained on the 4th and 10th day of the disease

Au antibodies

All serum samples in the investigation were also examined for antibodies against Au antigen In 2 persons antibodies were found Both were technicians at the department of dialysis None of them had had clinical hepatitis both had often been in contact with infected material

DISCUSSION

It is not possible to say how this epidemic of hepatitis had arisen Several possible explanations may however be suggested The patients received on the average two bottles of packed red cells per month during treatment with dialysis The use of such large amounts of blood naturally implies a considerable risk of infection via the blood per 'used Investigations have shown that Au itive blood used for transfusions often ed hepatitis in the recipients (*Okochi & Murakami* 1968 *Gocke & Kavey* 1969) On examination of 3 000 units of blood from the Blood Bank in Lund during 1969 4 (frequency about 0 1 per cent) were Au positive Another possibility is that the source of infection was introduced to the clinic by some patient

Immediately after the first few cases various precautionary measures were taken The epidemic nevertheless continued for 3 years and has not yet stopped This show he high infectious nature of the agent an r that au ons were inadequate A e dia is difficult to prevent fection od owing to the natu of the work Since the majority of the patients receiving dialysis were chronic carriers of Au antigen and thereby presumptive carriers of infection it was obviously difficult to prevent the spread of infection The prophylactic use of gammaglobulin routinely did not prevent the spread among the staff or among the patients a result which is in good agreement with previous investigations (*Ringert & Nystrom* 1967)

It is clear how important it is to do everything to prevent the introduction of hepatitis at a dialysis unit One of the most important and first applications for diagnosis of Au antigen must be a routine testing of blood used at the dialysis units

The clinical picture of the patients differed from that of the members of the staff and from that of the relatives in the way described in previous reports (*London et al* 1969 *Nordenfelt & Kjellen* 1969) Since it may be assumed that the staff and the patients were infected by the same agent comparison between the groups is of special interest

The patients had a very mild clinical picture with only a small increase in transaminases and often without subjective symptoms In many of the patients hepatitis would not have been diagnosed if the blood had not been regularly checked The members of the staff and the relatives had a classical picture of hepatitis with marked increases in transaminases usually also increased bilirubin and with typical subjective symptoms such as weakness nausea etc

The 2 groups differed from one another also in the reaction in the Au test This has also been noted in previous examinations of smaller series (*London et al* 1969 *Nordenfelt & Kjellen* 1969 *Turner & Bruce White* 1969) In members of the staff and relatives i e previously healthy persons Au antigen occurred immediately after the clinical signs of hepatitis and disappeared with one exception within 12 days of the onset of the disease, already before the clinical signs had disappeared The results of the Au test in the members of the staff and relatives showed

that the occurrence of the antigen was only temporary In 16 (12 personnel and 4 relatives) blood samples had often repeatedly been obtained soon after onset Nevertheless no Au antigen could be demonstrated in 6 In the patients on the other hand, the antigen in the serum appeared much earlier as a rule 5 weeks before clinical signs of hepatitis Once Au antigen had been demonstrated the patients were as a rule chronic carriers

Blumberg et al (1968) have shown that patients with Down s syndrome and with lepra become chronic carriers of Au antigen A common denominator of these two groups, like the patients receiving dialysis might be impaired immunological capacity, which has been discussed by *London et al* (1969) An interesting elucidation of this theory is the few cases in the patients and members of the staff who deviated from the typical pattern described above In the group of members of the staff there was one exception regarding the temporary occurrence of Au antigen This woman was a carrier of antigen for 6 months She was treated with cortisone Her condition as a carrier might have been due to immunosuppression because of steroid treatment In 2 of the patients Au antigen occurred in the serum temporarily in association with hepatitis in a corresponding way as in the group of staff members Their hepatitis was much more severe shown *inter alia* by a significantly higher GPT level The explanation to the different reaction in these two patients is not clear They may either have had a normal immunological capacity or they may have had a more intense antigen stimulation

A comparison could also be made regarding GPT determinations and Au test in the diagnosis of hepatitis As far as patients receiving dialysis are concerned the value of demonstrating Au antigen is obvious Since the antigen occurs already 5 weeks before other signs of hepatitis measures may be taken to prevent spread earlier than otherwise If the increase in GPT is only slight and the diagnosis of hepatitis therefore uncertain demonstration of Au antigen may confirm the diagnosis In the one case where the appearance of Au antigen in the serum was not followed by clinical hepatitis one relative fell ill In some cases Au antigen occurred during increased GPT probably because hepatitis occurred in an already damaged liver In one of the cases the patient had a chronically increased GPT which was regarded as being due to an injury by azathioprine treatment after previous attempted kidney transplantation In 3 patients with an increased GPT the increase was ascribed to hepatitis though no Au antigen could be demonstrated Owing to the close association between the occurrence of Au antigen and hepatitis in the remaining patients a search was made for some other cause of the increased GPT but in vain

In normals *i e* patients without chronic renal disease, it is more difficult to demonstrate the antigen because its occurrence in the blood is so transient Summing up examination of the blood for Au antigen proved a useful supplementary tool in the diagnosis of hepatitis and thanks to its specificity it is particularly useful in questions of a differential diagnosis

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We thank Mrs *E Miller* for skilful technical assistance

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SURFACE IMMUNOGENS OF *MYCOBACTERIUM TUBERCULOSIS* DEMONSTRATED BY IMMUNOFLUORESCENCE

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Under mild conditions saline extraction of tubercle bacilli produced an immunogenic mixture which in rabbits gave rise to precipitating sera with high fluorescence antibody (FA) titre Some purification of the major antigens was achieved by means of ion exchange chromatography and disc electrophoresis in polyacrylamide At least two immunogenic substances were found each with a different type of immunospecificity The one gave sera that stained *M tuberculosis* and BCG exclusively and by means of enzymatic digestion this was shown to be of protein nature

The existence of relatively immunospecific antibodies was demonstrated during the course of studies on circulating antibodies in experimental infections by means of immunofluorescence (1 2 3) During work with mammalian mycobacteria various problems arose that required further investigation (3) The fact that smears optimal for the fluorescence antibody (FA) reaction could be obtained with acetone fixation suggests that the surface antigens were non lipid in character Since killed bacilli in contrast to viable bacilli were not able to provoke the production of antibodies with FA reactivity the amount of immunogen might be minute or in a labile state Furthermore the low frequency of cross reaction with *M fortuitum* points to the existence of several immunogenic complexes on the surface of *M tuberculosis*

Preliminary experiments revealed that saline extracted *M tuberculosis* could not serve as substrate in a FA reaction with a FA positive serum in contrast to bacilli extracted with acetone Furthermore it was found that a saline extract was able to inhibit the homologous FA reaction in contrast to acetone extract which had no inhibitory effect

This paper reports the results of some attempts to isolate immunogens from the surface of *M tuberculosis* The character of the immunogenic substances is examined on the basis of the antibodies elicited by them

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MATERIAL AND METHODS

M tuberculosis (strain No SSC 879) was grown on fluid Sauton medium for 6 weeks before the addition of 0 5 per cent phenol The mass of bacilli was removed by filtration through paper washed twice on the filter with phosphate buffered saline (PBS 0 01 M phosphate 0 15 M NaCl pH

7.35) and finally suspended in about six volumes of PBS. The batch used in this study consisted of about 1 kg of bacilli grown in 18 l of medium.

Saline extraction of bacilli. The saline suspension was incubated at 37° C for 48 hours and then centrifuged at 3000 r.p.m. for 30 minutes. Proteins in the supernatant were precipitated with 80 per cent saturated ammonium sulphate, filtered through paper, dissolved and reprecipitated twice. The final precipitate was dissolved in and dialysed against PBS and then ultracentrifuged at 44 000 G for 8 hours (4). Three fractions were visible consisting of a lipoid surface layer, a clear supernatant and a translucent gelatinous pellicle. The clear supernatant was filtered through Millipore 0.2 μ membranes yielding 120 ml, the protein content being 5 mg/ml as measured by spectrophotometry at 280 nm.

Preparation of concentrated culture filtrate. Ammonium sulphate was added to the filtrate to 80 per cent saturation point. The precipitates were collected by filtration through paper, then dissolved and reprecipitated as mentioned above. Ultracentrifugation of the dissolved dialysed precipitate revealed three fractions which apparently were the same as those found in saline extract. The clear supernatant was filtered through Millipore 0.2 μ membranes yielding 200 ml, the protein content being 5 mg/ml.

Ion exchange chromatography of saline extract. Column chromatography was performed on DEAE Sephadex (Pharmacia, Uppsala, Sweden) using either 0.1 M TRIS-HCl (pH 8.4) or 0.05 M phosphate (pH 7.3) as initial buffer. The proteins were eluted by a linear gradient of saline. The results of an experiment with TRIS buffer are shown in Fig. 3.

In order to improve the resolution of the chromatographic method, equal amounts of saturated ammonium sulphate solution were added to all tubes in the fraction collector after the completion of a run. Fractions were pooled according to the results of immuno-electrophoretic examination of both supernatants and precipitates from the individual tubes.

Zone electrophoresis in polyacrylamide gel (disc electrophoresis). The apparatus designed by *Stelos* (10) was assembled from ordinary laboratory equipment and used as in the method described by *Davis* (5), modified only by increasing the amounts of the reagents used. The sample consisted of 30 mg of saline extract of *M. tubercul*... Electrophoresis was performed with a curre... f 10 mA at 4° C for 10 hours. The gel colu... was removed and a longitudinal slice cut ... staining with amido-schwarz. The remainder of ... column ...m...ately frozen in solid car... dioxide ... till frozen, was cut with a ...alpel ac... to ...e zones appearing on the s...ned slide. The gel segments were ground with a glass rod and the proteins were recovered with PBS.

Enzymatic digestion. Three portions of saline extract, each corresponding to 5 mg of protein, were digested with Crystalline Porcine Trypsin®, Crystalline Subtilisin® and Crystalline Bacterial α-Amylase® (Novo, Copenhagen) respectively. Each portion was digested with 5 μg enzyme for 24 hours at 37° C, emulsified without further treatment in Freund's adjuvant and injected into one rabbit.

Diffusion in gel a.m. Ouchterlony. This was performed as described by *Lind* (6).

Immuno-electrophoresis was carried out with an LKB apparatus according to the specification of the suppliers.

Immunization of rabbits. In all cases the immunization schedule consisted of one injection of the immunogenic solution in question emulsified in Freund's incomplete adjuvant. The amounts injected were as follows:

- Saline extract and culture filtrate: 1, 5 and 20 mg protein.
- Chromatographic fractions: 0.4–0.7 mg protein.
- Electrophoretic fractions: 1 mg (except for one fraction, where 0.5 mg was injected).
- Enzymatic digestion: corresponding to 5 mg protein before digestion.

Bleeding. Blood samples were taken from the marginal ear vein 3, 5 and 7 weeks after immunization. After 7 weeks the animals were bled by heart puncture. When sera were collected, EDTA was added to 0.5 per cent. FA titrations were performed with all serum samples. The FA immunospecificity investigations, immune diffusions and immuno-electrophoresis examinations were carried out with the sera collected after 7 weeks.

Protein determinations. All protein determinations were estimated by ultraviolet spectrophotometry at 280 nm, using rabbit serum globulin as standard.

Fluorescence antibody reacti... Standard smears were used to standardize the titrations. The homologous strain was grown on plates containing Löwenstein-Jensen medium. Colonies were emulsified in PBS in a mortar and the suspension was centrifuged at 1000 and 9000 r.p.m. for 15 minutes. The bacilli present in the supernatant after the first centrifugation but spun down during the second were suspended in PBS at a density of about 0.1 mg/ml. In this way clumps, cytoplasmic contents and cell ghosts were discarded. Phase contrast microscopy revealed the presence of unicellular organisms only. A large number of smears were then made on slides precoated with gelatine (3), fixed in acetone and stored at −70° C. The smears were thawed in acetone at room tempera-

ture before use as substrate in the indirect FA reaction as described previously (3)

RESULTS

Immunization with culture filtrates and saline extract Figs 1 a b shows the curves for the FA titres from rabbits immunized with three different doses of saline extract and culture filtrate It will be seen that both preparations gave rise to FA reactive sera with comparable titres and that the highest titres were developed with an immunization dose corresponding to 5 mg protein for both substances However, the smears stained by antisaline extract serum showed less background staining and more brilliant bacilli

Fig 2 a shows the result of an immunodiffusion precipitation experiment with the two types of sera obtained after 7 weeks Both preparations gave precipitating sera The antisaline extract serum caused at least four precipitation bands against both the homologous antigen and the culture filtrate One of the dense lines in this system (indicated by an arrow) showed identity with a line in the culture filtrate system lying close to the antibody reservoir The antibodies responsible for those lines are designated abI and the corresponding antigen agI Fig 4 gives the corresponding immuno electrophoretic patterns of the two sera against saline extract (Fig 4 s e and c f)

Column chromatographic fractionation of saline extract Fig 3 shows a typical ion exchange chromatography run on DEAE Sephadex in TRIS HCl NaCl buffer (pH 8 4) As a result of fractionation five fractions were obtained and injected in Freund s adjuvant into rabbits Table 1 shows the FA titres obtained and Fig 4 1–5 the immuno electrophoretic patterns using the sera against saline extract as antigen Fraction 1 and particularly Fraction 2 gave sera with high FA titres Their immuno electrophoretic patterns were dominated by a dense arc The corresponding dense line showed identity with the agI abI complex by immunodiffusion analysis (not shown in figures) As mentioned some experiments were carried out with phosphate buffer (pH 7 3) in DEAE Sephadex chromatography Fig 4 also shows the result of the immuno electrophoretic investigation of the serum produced by injection of the first fraction eluted (Fig 4 Ph I) The line corresponding to the agI abI complex is only just visible thus indicating a low concentration of abI in this serum

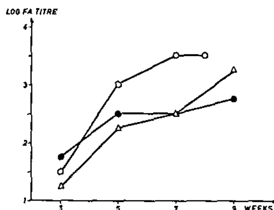


Fig 1a FA titres of sera from rabbits immunized with saline extract of *M tuberculosis*

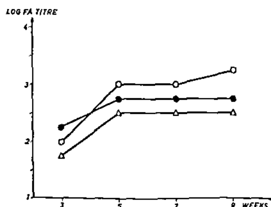


Fig 1b FA titres of sera from rabbits immunized with culture filtrates of *M tuberculosis* Each point represents the logarithmic mean of the titres from two rabbits

●——● 20 mg protein per rabbit
○——○ 5 mg protein per rabbit
△——△ 1 mg protein per rabbit

Disc electrophoresis of saline extract The gel column was cut into ten segm[illegible] 2 shows the amount of protein [illegible]

TABLE 4 *Specificity of Different Sera in Indirect FAT*

	ATCC* no.	Serum produced by immunization with					
		Saline extract	Culture filtrate	Chromatographic Fr 2	Disc electrophoretic		
					Fr 2	Fr 5	Fr 6
M avium	15769	—	—	—	—	—	—
M intracellulare	15985	—	—	—	+	—	—
M kansasii	12478	—	—	—	—	—	—
M marianum	19275	—	—	—	—	—	—
M bovis	19210	—	—	—	+	(+)	—
M bovis (BCG)	19274	(+)	+	(+)	+	+	+
M marinum	927	—	—	—	—	—	—
M *fortuitum*	6841	+	+	—	+	+	—
M smegmatis	14468	—	—	—	—	—	—
M phlei	19249	—	—	—	+	—	—
M vaccae	15483	—	—	—	—	—	—
M xenopi	19976	—	—	—	+	—	—
M microti	19422	—	—	—	+	—	—
M aquae	19977	—	—	—	+	—	—
M tuberculosis (11 strains†)		+11/11	+11/11	+11/11	+11/11	+11/11	+11/11
M bovis (9 strains†)		+0/9	+0/9	+0/9	+9/9	+7/9	+0 9

* Issued as Series I by International Working Group on Mycobacterial Taxonomy (IWGMT)
† Isolated in the Tuberculosis Department Statens Seruminstitut Copenhagen

plex was not found in the zone of reaction but a certain asymmetry in the agI abI band in the reference system might indicate the existence of a low concentration of abI in the subtilisin system As shown in Fig 2 d digestion with amylase had no effect on the immunodiffusion pattern Figs 4 s and 1 show the immuno electrophoretic pattern when the two sera had diffused against saline extract

Immunospecificity comparisons of the sera obtained Table 4 gives the results of the FA reactions when some of the sera were used against a variety of mycobacterial strains The sera obtained by immunization with saline extract and culture filtrate showed the same pattern of immunospecificity All strains of *M tuberculosis* *M bovis* (F G) and *M fortuitum* were stained but *M* *ovis* was not stained at all The second chromato graphic fraction gave serum which stained all strains of *M tuberculosis* and the BCG strain but none of the others In contrast the second electrophoretic fraction stained most of the species examined including *M intracellulare* *M phlei* *M xenopi* *M microti* *M aquae* and all strains of *M tuberculosis* and *M bovis* The fifth electrophoretic fraction gave serum which stained *M tuberculosis* *M bovis* (including BCG) and *M fortuitum* and the sixth fraction gave serum that stained *M tuberculosis* and BCG

DISCUSSION

When tubercle bacilli are stained by means of immunofluorescence the appropriate antibodies must be coupled to corresponding antigens at the surface of the bacillus This study shows that it is possible to extract immunogens from the surface of the bacillus under mild conditions and to use the sera

produced by these immunogens in FA tests. The existence of a very delicate outer electron dense layer was discussed in a study of the ultramorphology of the mycobacterial cell wall (7). This layer seemed to be of protein nature, at any rate the acid phosphatase was localized there. Therefore a mild extraction procedure might be sufficient for the removal of that layer. If more drastic means were used, there would obviously be a risk of extracting more deeply lying substances and even cytoplasmic constituents. *Parlett & Youmans* (8) used living cultures as antigen reservoirs in a modified agar diffusion precipitation test and found an increased number of precipitation lines as compared with those given by culture filtrates. This could perhaps be explained by release of the superficial antigen layer. *Thurston et al* (11) also used saline and aqueous extract in serological investigations obtaining successful result.

It was found in the present study that variation of the immunization dose from 1 to 20 mg of protein had no significant influence on the FA titres. Investigations with even smaller doses would be necessary to determine the optimal immunization dose.

Previous papers (1, 2, 3) reported the production of antibodies in rabbits by infection with living mycobacteria. The FA titres and the immunospecificities of these sera resembled those of the sera described in the present study but the main difference is the content of precipitins. As might be expected antiserum produced against soluble immunogens precipitates readily with extract and culture filtrate. However using the same technique and consequently the same level of sensitivity we have never been able to demonstrate precipitating antibodies in serum from rabbits injected with living mycobacteria. *Lind* (6) has reported the existence of a precipitin response in some sera produced after infection. However there must be a qualitative difference in the antibodies from the two kinds of sera.

The fractionation of the saline extract described in this paper revealed that different immunogens can give FA reactive sera. At least two different patterns of immunospecificity were found. The first group of antibodies which by immunodiffusion analysis was shown to contain the abI agI complex and might be due to a protein substance from the extract stained only *M tuberculosis* and BCG but none of the other mycobacterial species examined hitherto. The second group stained most of the mycobacterial species examined. The practical consequence of this specificity is a standardized production of sera for immunofluorescent typing of *M tuberculosis* (and BCG).

By injecting a fraction of saline extract it was possible to obtain an antiserum with a broad specificity as compared to the relatively narrow specificity of the crude saline extract. The reason might be that one of several immunogens was concentrated during the fractionation procedure. In the FA modification used in the present study all specificity determinations were carried out at a dilution equal to the penultimate titration stage thus ensuring that antibodies due to minor immunogens would not be observed.

Differences in materials and methods complicate comparison with antigenic substances described previously in the literature. In a series of publications *Yoneda et al* (12) described the isolation of two pure fractions from culture filtrates viz. α and β antigens. The immunogen described in the present study is not identical with either of these since it is not precipitated by 50 per cent saturation of ammonium sulphate in contrast to the α and β antigens. Furthermore the immunospecificity of the serum produced is more narrow, only *M tuberculosis* and BCG being stained.

Of the three purification methods used the ion exchange chromatography combined with ammonium sulphate precipitation yielded a well purified AgI. However disc electrophoresis yielded the substance which cross reacted with all mycobacteria. Other investigators emphasize the better resolution of mycobacterial antigens by means of disc electrophoresis (9). These observations have not

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M marianum	19275	—	—	—	—	—	—
M bovis	19210	—	—	—	+	(+)	—
M bovis (BCG)	19274	(+)	+	(+)	+	+	+
M marinum	927	—	—	—	—	—	—
M fortuitum	6841	+	+	—	+	+	—
M smegmatis	14468	—	—	—	—	—	—
M phlei	19249	—	—	—	+	—	—
M vaccae	15483	—	—	—	—	—	—
M xenopi	12976	—	—	—	+	—	—
M microti	19422	—	—	—	+	—	—
M aquae	19277	—	—	—	+	—	—
M tuberculosis (11 strains†)		+11/11	+11/11	+11/11	+11/11	+11/11	+11/11
M bovis (9 strains†)		+0/9	+0/9	+0/9	+9/9	+7/9	+0/9

* Issued as Series I by International Working Group on Mycobacterial Taxonomy (IWGMT)
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plex was not found in the zone of reaction but a certain asymmetry in the agI abI band in the reference system might indicate the existence of a low concentration of abI in the subtilisin system As shown in Fig 2d digestion with amylase had no effect on the immunodiffusion pattern Figs 4 5 and 6 show the immuno electrophoretic pattern when the two sera had diffused against saline extract

Immunospecificity comparisons of the sera obtained Table 4 gives the results of the FA reactions when some of the sera were used against a variety of mycobacterial strains The sera obtained by immunization with saline extract and culture filtrate showed the same pattern of immunospecificity All strains of *M tuberculosis* *M bovis* (BCG) and *M fortuitum* were stained but *M bovis* was not stained at all The second chromatographic fraction gave serum which stained all strains of *M tuberculosis* and the BCG strain but none of the others In contrast the second electrophoretic fraction stained most of the species examined including *M intracellulare* *M phlei* *M xenopi* *M microti* *M aquae* and all strains of *M tuberculosis* and *M bovis* The fifth electrophoretic fraction gave serum which stained *M tuberculosis* *M bovis* (including BCG) and *M fortuitum* and the sixth fraction gave serum that stained *M tuberculosis* and BCG

DISCUSSION

When tubercle bacilli are stained by means of immunofluorescence the appropriate antibodies must be coupled to corresponding antigens at the surface of the bacillus This study shows that it is possible to extract immunogens from the surface of the bacillus under mild conditions and to use the sera

produced by these immunogens in FA tests The existence of a very delicate outer electron dense layer was discussed in a study of the ultramorphology of the mycobacterial cell wall (7) This layer seemed to be of protein nature, at any rate the acid phosphatase was localized there Therefore, a mild extraction procedure might be sufficient for the removal of that layer If more drastic means were used, there would obviously be a risk of extracting more deeply lying substances and even cytoplasmic constituents *Parlett & Youmans* (8) used living cultures as antigen reservoirs in a modified agar diffusion precipitation test and found an increased number of precipitation lines as compared with those given by culture filtrates This could perhaps be explained by release of the superficial antigen layer *Thurston et al* (11) also used saline and aqueous extract in serological investigations obtaining successful result.

It was found in the present study that variation of the immunization dose from 1 to 20 mg of protein had no significant influence on the FA titres Investigations with even smaller doses would be necessary to determine the optimal immunization dose

Previous papers (1 2 3) reported the production of antibodies in rabbits by infection with living mycobacteria The FA titres and the immunospecificities of these sera resembled those of the sera described in the present study but the main difference is the content of precipitins As might be expected antiserum produced against soluble immunogens precipitates readily with extract and culture filtrate However using the same technique and consequently the same level of sensitivity we have never been able to demonstrate precipitating antibodies in serum from rabbits injected with living mycobacteria *Lind* (6) has reported the existence of a precipitin response in some sera produced after infection However there must be a qualitative difference in the antibodies from the two kinds of sera

The fractionation of the saline extract described in this paper revealed that different immunogens can give FA reactive sera At least two different patterns of immunospecificity were found The first group of antibodies which by immunodiffusion analysis was shown to contain the abI agI complex and might be due to a protein substance from the extract stained only *M tuberculosis* and BCG but none of the other mycobacterial species examined hitherto The second group stained most of the mycobacterial species examined The practical consequence of this specificity is a standardized production of sera for immunofluorescent typing of *M tuberculosis* (and BCG)

By injecting a fraction of saline extract it was possible to obtain an antiserum with a broad specificity as compared to the relatively narrow specificity of the crude saline extract The reason might be that one of several immunogens was concentrated during the fractionation procedure In the FA modification used in the present study all specificity determinations were carried out at a dilution equal to the penultimate titration stage thus ensuring that antibodies due to minor immunogens would not be observed

Differences in materials and methods complicate comparison with antigenic substances described previously in the literature In a series of publications *Yoneda et al* (12) described the isolation of two pure fractions from culture filtrates *vi* α and β antigens The immunogen described in the present study is not identical with either of these since it is not precipitated by 50 per cent saturation of ammonium sulphate in contrast to the α and β antigens Furthermore the immunospecificity of the serum produced is more narrow only *M tuberculosis* and BCG being stained

Of the three purification methods used, the ion exchange chromatography combined with ammonium sulphate precipitation yielded a well purified AgI However disc electrophoresis yielded the substance which cross reacted with all mycobacteria Other investigators emphasize the better resolution of mycobacterial antigens by means of disc electrophoresis (9) These observations hav

TABLE 4 *Specificity of Different Sera in Indirect FAT*

	ATCC* no	Serum produced by immunization with					
		Saline extract	Culture filtrate	Chromatographic Fr 2	Disc electrophoretic		
					Fr 2	Fr 5	Fr 6
M avium	15769	—	—	—	—	—	—
M intracellulare	15985	—	—	—	+	—	—
M kansasii	12478	—	—	—	—	—	—
M marianum	19275	—	—	—	—	—	—
M bovis	19210	—	—	—	+	(+)	—
M bovis (BCG)	19274	(+)	+	(+)	+	+	+
M marinum	927	—	—	—	—	—	—
M fortuitum	6841	+	+	—	+	+	—
M smegmatis	14468	—	—	—	—	—	—
M phlei	19249	—	—	—	+	—	—
M vaccae	15483	—	—	—	—	—	—
M xenopi	19976	—	—	—	+	—	—
M *microti*	19422	—	—	—	+	—	—
M aquae	19277	—	—	—	+	—	—
M tuberculosis (11 strains†)		+11/11	+11/11	+11/11	+11/11	+11/11	+11/11
M bovis (9 strains†)		+0/9	+0/9	+0/9	+9/9	+7/9	+0/9

* Issued as Series 1 by International Working Group on Mycobacterial Taxonomy (IWGMT)
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TABLE 1 *Results of MLC between*

Exp no	1	2	3	4	5	6	7	8	9	10
Cell A Donors B K*	OL MA BR(1)	OL AN BR(1)	AN MA BJ(2)	FI JO TH(1)	TH SO AS(2)	ZE GR EA(1)	LE HL HA(1)	UH KB NB(1)	OT IO LI(1)	BD TO KE(2)
A + Bm	508	263	65	176	386	106	298	276	297	464
B + Am	568	424	124	181	297	55	359	234	342	391
A + Km	4048	1160	10263	3366	12760	2763	14317	6045	6341	17355
B + Km	4009	1806	6411	5409	11477	2167	11087	6054	6660	13094
K + Am	4866	1142	7732	4305	4629	879	14489	6681	10293	13536
K + Bm	5663	1529	6240	5068	4123	1032	11588	6604	10784	11957
A + Am	393	214	171	200	373	240	279	291	203	450
B + Bm	443	373	158	142	333	86	591	189	353	375
K + Km	1316	317	50	338	210	208	327	252	197	238
Am + Km	105	118	98	48	134	131	155	101	115	489
Bm + Km	129	131	95	69	112	85	197	124	–	583
A + PHA	27197	28422	18218	25187	16255	5092	14605	16308	19210	20565
B + PHA	25135	32160	6610	12252	19226	8972	15286	21460	16350	23578

* Numbers in brackets indicate number of haplotypes for which cell donor K differed from the HL-A identical siblings A and B

In the remaining 84 HL-A non identical mixtures the lowest ratio between mixtures and controls found was 3.70. This indicates that the method described permits a clear distinction between HL-A identical and HL-A non identical cell donors.

In the mixtures of K + Am and K + Bm the responding cells are stimulated by the same HL-A antigens. To compare the variation seen in these pairs of responses with the technical variation of the method 27 experiments were performed where a single blood sample was divided into two parts, prepared separately and finally used to stimulate cells from another normal cell donor. Analysis of variation showed no difference in the variation obtained by stimulation with cells from two parts of the same blood sa[mple] or from two HL-A identical siblings (experiments including only normal cell donors (n= [illegible])). However in experiments Nos 11–17 where uraemic cell donors were included the variation was clearly higher suggesting that these values belonged to a different population (P < 0.01). Therefore these experiments are excluded from the following calculations.

For each pair of responses the coefficient of variation can be calculated after subtraction of the unstimulated control. The highest value found by stimulation with cells from two HL-A identical siblings was 29 per cent as compared to 39 per cent by stimulation with cells from two parts of the same blood sample.

From the results in Table 1 mean values for stimulation in different genetic combinations can be calculated.

In the 32 one haplotype differing mixtures the mean stimulation after subtraction of the relevant unstimulated controls was calculated to 4970 CPM. In the 24 two haplotype dif

'L A *Identical Siblings*

11	1?	13	14	15	16	17	18	19	?0	21
BE TO JØ(1)	JØ AG BI(1)	KA ER KN(1)	KA RI KN(1)	AR ER HA(1)	GU MO u r	SU RI FR(2)	HE FR EW(2)	RI EW FR(2)	FR SA EW(?)	KJ PU AS(1)
?16 565	1034 485	501 332	122 250	325 313	226? 712	577 414	368 334	406 330	186 319	208 87
3630 4555	12368 8773	7212 5556	4863 2104	1145 1761	15062 16317	6675 10805	10?08 10418	8464 8222	4083 63??	1780 1178
6318 4356	13745 10643	5167 2855	5404 2130	902 1581	16816 11732	5?34 5116	12554 14663	9664 7360	?59? ?? ?	?6? 41 ?
399 250 410	1061 328 897	647 203 287	294 122 278	195 386 176	1438 485 860	491 322 122	293 3?4 309	476 353 255		
438 495	?64 164	336 120	160 90	76 97	364 184	204 167	583 465	280 262		
22562 20572	27726 29223	29987 21483	– –	28609 23895	16703 25154	19913 23539	10047 9735	16852 19000	1	

For symbols see text

fering sibling mixtures this value was 9200 CPM These results may be compared with the mean stimulation found in 40 mixtures of randomly chosen unrelated cell donors of 8700 CPM (accompanying paper)

DISCUSSION

The results of the MLC experiments performed fall into two clearly separable groups The *HL A identical* group *shows non sti*mulation (the ratio between mixtures and controls being 1 13 ± 0 44 (s d)) and HL A disparate group shows unequivocal stimulation (the lowest ratio found being 3 70) The correlation between MLC and serotyping for HL A antigens has been complete in siblings *Bach et al* (2) have published one case of stimulation between two serotypically identical siblings but as these investigators point out this *could be due to recombination within the HL-A region not detectable wi* th sera used In view of the high numbe of serotypically identical siblings showing non stimulation in MLC reported until now (1 19) it seems very unlikely that minor loci antigens alone may cause stimulation in human MLC Furthermore the clear separation between HL A identical and HL A non identical mixtures speaks against the possibility of low grade stimulation due to minor *loci antigens*

The role of ABO antigens is well established in transplantation (7) The question is whether ABO as a histocompatibility system is to be classified as strong or weak The experiments performed gives no indication of stimulation due to ABO antigens in MLC By this criterion therefore t ABC system must be classified as weak

Bernan & McKhann (3) have sho intraperitoneally injected spleen cel

TABLE 2 *Results of ABO and HL-A Typing of the Cell Donors*

Family	Exp no	Cell donor	Age	Sex	ABO type	HL-A type	Parental HL-A haplotypes
1	1 2 3	OL	29	♂	A	3 UPS/2	bd
		MA	22	♀	O	3 UPS/2	bd
		BR	23	♀	A	1 7/2	ad
		AN	25	♀	O	3 UPS/2	bd
		BJ	20	♂	A	1 7/Bax 12	ac
2	4 5	FI	31	♂	A	Bax 12/2 12	bc
		JO	29	♀	A	Ba 12/2 12	bc
		TH	42	♂	A	Bax 12/2 FJH	bd
		SO	36	♀	A	Bax 12/2 FJH	bd
		AS	45	♀	A	13/2 12	ac
3	6	ZE	48	♀	A	1 7 8	
		GR	46	♀	A	1 7 8	
		EA	40	♀	A	1 2 8	
4	7	LE	28	♀	A	1 7/3 AJ	ad
		HI	44	♂	O	1 7/3 AJ	ad
		HA	32	♀	O	1 7/1 8	ac
5	8	UH	60	♂	O	11 8/2 LND	ad
		KB	66	♂	O	11 8/2 LND	ad
		NB	63	♂	A	11 8/2 SL	ac
6	9	OT	31	♂	B	2/1 4A2	bc
		PO	41	♀	B	2/1 4A2	bc
		LI	37	♀	B	1 8/1 4A2	ac
7	10 11 12	BD	32	♀	A	1 5/11 Rx	ad
		TO	27	♂	AB	1 5/11 Rx	ad
		KE	36	♂	B	3 5/Bax 12	bc
		BE	22	♂	A	1 5/11 R	ad
		JØ(u)	29	♂	A	3 5/11 Rx	bd
		AG	26	♀	B	3 5/11 Rx	bd
		BI	33	♀	B	3 5/Bax 12	bc
8	13 14	KA(u)	21	♂	O	Bax LND/	ad
		ER	35	♂	O	Bax LND/	ad
		KN	33	♂	O	T12	bd
		RI	37	♀	O	Ba LND/	ad
9	15	AR(u)	29	♂	A	Li 12/1 8	ac
		ER	26	♂	A	Li 12/1 8	ac
		HA	20	♂	A	Li 12/	ad
10	16	GU(u)	46	♀	O	2 12 BB	
		MO	51	♂	A_1	2 12 BB	
		UR				–	
11	17 18 19 20	SU(u)	53	♂	B	2 7 8	
		RI	57	♂	B	2 7 8	
		FR	[illegible]	♂	B	1 3 8	
		HE	53	♂	B	1 3 8	
		EW	[illegible]	♂	B	2 7 8	
		SA	1	♂	B	1 3 8	
12	21	KJ	6	♂	O	2 LND/1 BB	bc
		PU	[illegible]	♂	A	2 LND/1 BB	bc
		AS	[illegible]	♀	O	1 BB/2 LND	cd

(u) Indicates uremic cell donor

failed to induce immunity to subsequent skin grafts when only weak antigens were involved However, if the injected cells were incompatible for both weak and strong antigens immunity was induced to the weak as well as to the strong antigens

McBride & Schierman have reported a similar finding (17) Immunization of chickens with red blood cells incompatible only with respect to antigens determined by the weak A blood group locus failed to induce antibody formation but if the injected cells were incompatible also for strong B blood group locus antigens antibodies were raised against both B and A antigens The strong B antigens acted as a carrier for the weak A antigens

Thus there are examples from both cellular and humoral immunity of increased response to weak antigens by simultaneous immunization to strong antigens The possibility was therefore considered that HL A antigens might act as a carrier for minor loci antigens in MLC Previous studies have shown that the MLC technique allows to distinguish between cell donors differing for one or two haplotypes the two haplotype differing mixtures within the single experiment consistently giving higher stimulation than one haplotype differing mixtures (26) A possible additive effect due to minor loci antigens therefore in advance was expected to be weak in comparison to the stimulation provoked by HL-A antigens In the experiments reported here no evidence for a contribution due to non HL A antigens was obtained The variation obtained by stimulation of a given responder with cells from two HL A identical siblings was not greater than the variation obtained by setting up the same mixture in two parallel experiments It may therefore be concluded that if minor loci antigens have an additive effect to HL A antigens this effect is so small that it is undetectable by the present technique This conclusion is also supported by the finding of equal mean stimulation in mixtures from two haplotype differing siblings and in mixtures from unrelated persons

In experiments including uraemic cell donors the variation found was clearly higher In 4 out of 6 experiments the mitomycin treated cells from the uraemic sibling caused stronger stimulation compared to the non uraemic HL A identical sibling However, the number of experiments must be extended before any conclusion can be drawn on this point

Accepting that the response in MLC is solely controlled by HL A determined antigens, any variation in the responses obtained by stimulation of HL A identical siblings with the same allogeneic cells must be due to technical variation, serum factors and possibly individual variation in lymphocyte responsiveness It has been shown that allogeneic cells and PHA both stimulate the thymus dependant lymphocytes in the peripheral blood (13) It was therefore hoped that PHA stimulation might function as a standard for reactivity in MLC However, as seen from Table 1 no correlation was found between stimulation of the HL A identical siblings with allogeneic cells and PHA

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		HE	[illegible]	♂	B	1 3 8	
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each child Therefore, if recombination within the HL A system has not occurred, only four different genotypes are possible in siblings and accordingly 25 per cent of all siblings must be HL-A identical Parent and child normally share only one haplotype but if the two parents have one haplotype in common, the possibility of finding children HL A identical with one or the other parent is present

Because the number of possible HL A genotypes in siblings excluding crossing over is limited to four it is highly probable that siblings are truly HL A identical if they show identical typing pattern in spite of limitations in the number and specificity of the available typing sera In all other combinations however the diagnosis of HL-A identity is completely dependent on the ability of the sera used unequivocally to define all HL A antigens involved

Nearly all antigens determined by lymphocytotoxicity and thrombocyte complement fixation techniques seems to belong to the two series LA and Four and although serological evidence for a third sublocus has been claimed (15 18) it is still a matter of debate whether additional subloci of the HL A system may be defined serologically No unequivocal evidence for the existence of sera defining HL A specificities outside the LA and Four series have so far been obtained in any of the Scandinavian laboratories involved in Scandiatransplant (4 13)

If the HL-A system only consisted of the two subloci mentioned above unrelated persons typing identical for four well defined HL A antigens *i e* two LA and two Four antigens should be truly HL A identical exactly as are HL A identical siblings

The mixed lymphocyte culture (MLC) technique has proved to be a reliable means for the diagnosis of HL A identity Reactivity in MLC is solely controlled by HL A antigens and it is possible using this technique to distinguish clearly HL A identical from HL A non identical cell donors (accompanying paper)

In a previous report 4 pairs of unrelated cell donors typing identical for 4 different supposedly well defined HL-A antigens were tested in MLC (7) None of the pairs were truly HL A identical In the present report this material is extended and the MLC responses obtained are compared with the results of MLC between family members

MATERIAL AND METHODS

If not otherwise stated all cell donors were healthy subjects who were HL A typed as panel donors or in connection with family studies One of the serotypically identical pairs (Experiment No 2) was included in the previous report (7)

HL A typing was performed using lymphocytotoxicity and thrombocyte complement fixation techniques (5 10) In addition to highly selected sera of well defined specificity varying numbers of multispecific sera were used Our thanks are expressed to dr *Kissmeyer Nielsen* Aarhus for retyping most of the serotypically identical cell donors In all cases full agreement between the typings in Aarhus and Copenhagen was obtained

Concerning the antigens Ba and La belonging to LA series and BB R FJH and LND belonging to Four series we refer to the studies of *Svejgaard et al* and of *Thorsby & Kissmeyer* (11 14) It has now been stated that anti FJH (Thorsby) contains two components one of which is present in AJ Sa (15) The other component is present in the sera 00004 and 001/0 (RH Copenhagen) used in this study All subjects in the present work who are FJH positive possess the component defined by the sera 00004 and 001/0

The SL serum too contains two components one of which is defined in this study by a serum JT (RH Copenhagen) The LA and Four identical persons in Experiment No 9 possessing this JT antigen are both of them panel donors and have always given identical reactions in all sera about 100 which contain a mixture of known and unknown specificities In the sera mentioned are not included those containing anti HL A 7 and 8

4A? which is present in one of the controls is a lowfrequent antigen defined by an antibody rather often present in sera containing anti HL A [illegible] and strongly cross reactive with this

Mixed lymphocyte cultures were prepared as described in the accompanying paper As culture medium TC 199 (Chas...) supplemented with 33 per cent serum pool from the donors included in the experiments was used To avoid a possible blocking effect by antibodies (3) sera from previously pregnant women and blood transfused individuals were not used

The cultures contained 1×10^6 responding lymphocytes and 2×10^6 mitomycin treated stimulating lymphocytes in a volume of 2 ml The proliferative response was assayed 90 hours after preparation of the cultures by the cellular uptake of ¹ C thymidine All cultures were harvested in triplicate and the results were expressed as CPM minus background per 1×10 responding lymphocytes of the initial culture

RESULTS

Table 1 shows the protocol of experiments and the results obtained In Experiments 1-10 A and B refer to serotypically identical unrelated cell donors, while K represents a randomly chosen cell donor included as positive control One way MLC s were prepared in all possible combinations between the three cell donors The mitomycin treated cells are indicated my m and accordingly A + Bm refers to a mixture of responding cells from A and stimulating cells from B Unstimulated controls were prepared by mixing responding cells with autologous mitomycin treated cells and as controls for the efficiency of the mitocycin treatment mix tures of HL A non identical mitomycin treated cells were prepared

In Experiment No 11 A and B refer to a uraemic patient and his father, respectively both typing identical for HL A 1 2 8 and 12 The mother was in this experiment included as the control subject

Table 2 shows the results of the HL A typing Besides being identical for the pe cificities shown, all the identical cell donors showed exactly the same typing pattern against a large panel of multispecific sera Where typing of family members to the test subjects allowed determination of HL A genotypes these are indicated i the t el The control subjects were chose r without their HL A type bei g k t advance These cell donors wer t spectively

As seen from Table 1 all m typically identical unrelatec showed significant stimulation ε trols, the ratio between mixt stimulated controls ranging ı 40 40 This result may be compa

TABLE 1 *Results of MLC between LA and Four Identical Cell Donors*

p No		1	2	3	4	5	6	7	8	9	10	11
donors	A B K	AJ KR PJ	RJ RE HJ	MK VA HW	SK AW CH	KH SK PN	MJ PG BR	IK MJ PS	KK NA HP	ER KL 1 O	AA HW SF	PA JA KA
+Bm +Am		5379 6007	4444 3178	4378 3411	3312 1160	3240 11715	6816 4757	8398 8352	1006 1945	1795 1291	792 1414	793 151
+Km +Km		14733 10475	5840 5342	8581 5058	7838 2187	4495 20297	6749 8575	14928 25083	2453 5064	10372 15099	2654 1965	4689 5655
+Am +Bm		10698 10565	10973 14941	6575 17863	5192 3822	12070 19615	6606 7136	15062 19189	7932 14722	2810 2591	5569 2562	4491 8301
+Am -Bm -Km		312 506 724	770 442 577	299 174 266	1277 412 [illegible]	319 2421 2917	226 553 321	208 763 433	105 137 114	266 325 148	219 148 240	186 158 328
+Km +Km		705 203	145 146	163 118	88 113	203 291	109 115	137 775	54 67	65 53	71 63	275 315

For symbols ee te t

ants recognized in the pairs may be different in detailed structure. These differences may be concealed by HL-A typing due to cross reactivity between these determinants while MLC might reveal such differences. In view of the great difficulty in defining mono specific sera against HL-A antigens due to cross reactivity between several of these antigens (6), this possibility cannot be excluded. However, the stimulation seen in Experiments 1, 4, 5 and 10 which included only some of the most well defined antigens and the fact that the cell donors besides being identical for well defined antigens typed identically for a large number of multi specific sera speaks against this explanation.

Finally, the results may be explained by the existence within the HL-A system of subloci additional to the "LA" and "Four" subloci. The stimulation seen between "LA" and "Four" identical unrelated would be due to incompatibility for these loci while siblings inheriting the complete haplotype in case of "LA" and "Four" identity, would be identical for the remaining HL-A subloci too.

If this explanation were true it might be postulated that reactivity in MLC was not determined by the "LA" and "Four" antigens but controlled instead by a MLC sublocus closely linked to the "LA" and "Four" subloci. However the relatively low stimulation between "LA" and "Four" identical unrelated subjects compared with randomly chosen cell donors argues strongly for the importance of "LA" and "Four" antigens in MLC (Table 1).

One of the main characteristics of strong transplantation antigens is their ability to induce antibody formation. The difficulty in demonstrating antibodies against other HL-A antigens than those determined by the "LA" and "Four" subloci could therefore argue against their existence or at least indicate that these antigens were weak. The MLC strength of the unspecified HL-A antigens however is rather strong, the stimulation seen between "LA" and "Four" identical unrelated being of the same order of magnitude as between siblings differing for one haplotype (Fig. 1).

Furthermore Table 3 shows how an unrelated "LA" and "Four" identical person repeatedly stimulated a responder to the same level as his HL-A 12 incompatible mother.

These results indicate that the unspecified HL-A antigens whether consisting of subcomponents of known HL-A antigens or determined by additional subloci of the HL-A system may have the same MLC strength as the "LA" and "Four" antigens known at present. Furthermore these findings explain the poor correlation usually found between number of incompatible HL-A antigens and stimulation in MLC (1, 12).

The present results are in complete agreement with *Thorsby's* recent demonstration of significantly longer mean survival time of skin grafts exchanged between "LA" and "Four" identical unrelated subjects (13.1 ± 2.14) compared to randomly chosen individuals (10.0 ± 1.55) while skin grafts exchanged between HL-A identical siblings survive considerably longer (20.0 ± 4.23) (13). Furthermore these results are in accordance with the correlation found between compatibility as revealed by HL-A typing and the results of renal transplantation in unrelated donor recipient pairs (2, 8, 9, 16).

In the experiments reported here and in the previous report (7) no HL-A identical pairs were found between unrelated individuals. However the lack of stimulation in mixtures between the "LA" and "Four" identical father and son (Experiment No. 11) demonstrates the possibility of finding unrelated subjects (the parents) sharing one haplotype and recently *van Rood* and *Eijsvoogel* have reported a single well documented case of non stimulation in MLC between two serotypically identical unrelated subjects (17).

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INCREASED SERUM LEVELS OF IgM IN ACUTE SALPINGITIS RELATED TO THE OCCURRENCE OF *MYCOPLASMA HOMINIS*

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The concentrations of IgG IgA and IgM were determined in serum specimens collected from women with acute salpingitis from women with infections confined to the lower genital tract and from healthy women Thirty four per cent of the acute salpingitis patients had a marked increase of the serum level of IgM but normal or only slightly increased serum concentrations of IgG and IgA *Mycoplasma hominis* was recovered from all except two of the salpingitis patients with increased levels of IgM Indirect haemagglutinating antibody to *M hominis* was found in all but one of these cases In those salpingitis cases that had serum levels of IgM within the normal range antibody to *M hominis* was found significantly less often

Iost workers agree that acute salpingitis is enerally caused by an infection ascending om the lower genital tract In a series of 50 lpingitis patients *Mycoplasma hominis* was ecovered from the cervix in 62 per cent of ie cases (*Mardh* & *Westrom* 1970 a) *M ominis* has also been isolated from the 'allopian tubes in women with acute salpin itis and a significant rise of the titre of anti ody to *M hominis* has been demonstrated n such cases (*Mardh* & *Westrom* 1970 b)

In healthy individuals the serum levels of he immunoglobulin classes IgG IgA and gM are remarkably constant (*Wilhelm* & *Villiams* 1965) and in the acut stage of most infectious diseases the serum levels of these immunoglobulin classes do not differ appreciably from those seen in health (*Zanussi* & *Medina* 1968) A marked selective increase of the serum level of IgM has been demonstrated in a few infectious diseases *inter alia* in *Mycoplasma pneumoniae* infections (*Fei* 1967)

The present study was undertaken in order to determine if patients with *M hominis* infections have a serum immunoglobulin pattern similar to that found in patients with *M pneumoniae* infections The serum levels of the immunoglobulin classes IgG IgA and IgM were determined in patients with acute salpingitis The finding of a marked increase of IgM in such patients is reported

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MATERIAL AND METHODS

Immunoglobulin antisera Rabbit antihuman IgG IgA and IgM antisera were used

Quantitations of IgG and IgA were made by Oudin s single diffusion tube technique (*Oudin* 1952) as modified by *Bachmann & Laurell* (19o3) The samples were diluted 1 2 in a purified 5 per cent human albumin solution The tubes were kept at room temperature and read after 23 and 24 hours for the quantitation of IgG and IgA respectively The results are given as the mean value of double determinations The standard error calculated from the double determinations which were performed on the same day was 21 and 6 mg per 100 ml for IgG and IgA respectively

Quantitation of IgM was with some modifications made according to the single radial diffusion technique described by *Mancini et al* (1965) The sera were mixed with an equal volume of 0 4 M 2 mercaptoethanol in saline and incubated at room temperature for 2 hours The mixtures were diluted 1 10 and 1 50 in barbital buffer pH 8 6 From each of these dilutions double analyses were made on different plates on the same day The diameters of the precipitation zones were read after 24 hours incubation at room temperature The results are given as the mean value of the four determinations The standard error calculated from the double determinations was 1 7 mg per 100 ml when the samples were tested at a dilution of 1 10 and 6 2 mg per 100 ml when diluted 1 50

A pool of serum from 120 healthy blood donors was included on each test occasion as a standard The concentrations of IgG IgA and IgM in this serum pool were 1050 198 and 77 mg per 100 ml The range for IgG IgA and IgM in the sera comprising the standard serum pool was 685 to 1680 100 to 315 and 25 to 155 mg per 100 ml respectively The normal range used in the following refers to these latter values

The indirect haemagglutination technique used for the demonstration of antibody to *M hominis* has been described earlier (*Mardh & Westrom* 19/0 b) as well as the sampling technique and the method for cultivating *M hominis* and bacteria (*Mardh & Westrom* 1970 a)

The complement fixation test with *M pneumoniae* was performed with a microtitre system (Flow Laboratories Ltd) The *M pneumoniae* antigen was obtained from Microbiological Associates Bethesda Maryland U S A Two units of complement were employed with overnight fixation at 4 C

Cold agglutinin were assayed with group 0 adult as well as group 0 cord blood cells using a 0 1 per cent solution of the erythrocytes Twofold serum dilutions were m ed with an equal volume of the erythrocyte susp n ion The reading were made macroscopically fter overnight incubation at 4 C

The *Wassermann & Meinicke* reactions were performed according to the standard techniques of this laboratory

Sera were stored at —20 C until used

One hundred and sixty six serum specimens from 93 women were investigated These women were from 16 to 29 years old and the age distribution within the three groups presented below was similar None of the investigated women were pregnant

Patients with acute salpingitis (Group I) The diagnosis was confirmed by laparoscopy in all 53 cases According to the laparoscopic findings the degree of the inflammatory changes in the adnexa was classified as stage A B or C In stage A there were reddened serosa oedema of the tubal wall and purulent discharge from the abdominal orifices of the tubes In stage B there were more advanced inflammatory changes—such as swe ing of the tubal wall closure of the abd minal fic s and deposits of fibrin on the sero surfac In stage C there was abscess forma or or r ivic peritonitis From all patients ser e e c ted on the day of admission to the c ic h as also the day of laparoscopy Co utive erum specimens were collected from 3 e p nt All the salpingitis cases had sig ct infection in the lower genital tract No treatment had b en given before the first serum speci en was drawn

Patients with infections confin to th lower genital tract (Group II) This gr compri ed 20 women with signs of infection in the lower genita tract The diagnostic criteria were those r ported earlier (*Mardh & Westrom* 1970 a) The women had had symptoms of a lower genital tract infection for at least 2 weeks before sera were drawn Consecutive serum specimens were collected from 10 of the patients

Healthy females (Group III) The group comprised 20 women in whom the gynaecological examination revealed normal conditions Con ecut e serum pecimens were collected from 10 of them

Cultural and serological f ndings as to *M hominis* in 38 15 and 12 of the women in Group I II and III respectively have been reported previously (*Mardh & Westrom* 1970 b)

RESULTS

Immunoglobulin Levels in Patients with Acute Salpingitis

Eighteen of the 53 salpingitis patients or 34 per cent had increased serum levels of IgM in one or more specimens (T ble 1) while in the remaining 35 cases the serum levels of IgM were within the normal range (25 to 155 mg per 100 ml) Concentrations

TABLE 1 *The Serum Concentration of IgG, IgA and IgM in Salpingitis Patients with a Serum Concentration of IgM > 155 mg per 100 ml*

Case no	Age	Stage*	Day†	IgG	IgA (mg per 100 ml)	IgM
1	16	A	0	1197	301	285
			9	1386	313	380
			40	1176	214	343
2	23	C	0	1806	230	315
			14	1848	230	305
3	19	B	0	1328	230	300
			5	1208	206	320
			12	1428	226	325
			19	1218	198	245
			35	1134	218	245
4	26	B	0	945	129	260
			28	1554	230	315
5	29	B	0	785	143	200
			7	939	190	260
6	22	B	0	1113	194	131
			20	1932	316	243
7	25	A	0	1155	267	175
			7	1890	404	230
8	19	B	0	1260	166	194
			13	1806	226	210
9	23	A	0	786	154	175
			9	786	162	210
10	17	B	0	1029	129	148
			14	1050	139	149
			35	1365	143	235
11	21	B	0	1092	182	118
			34	1281	182	145
			47	1302	218	235
12	23	B	0	945	194	147
			9	1260	222	240
			28	1302	174	215
13	21	A	0	798	31[illegible]	3[illegible]5
14	18	C	0	1890	30[illegible]	3[illegible]0
15	21	B	0	1260	[illegible]	305
16	20	A	0	1092	[illegible]	[illegible]0
17	18	B	0	1113	[illegible]	[illegible]0
18	19	A	0	1218	[illegible]	[illegible]15

* The degree of the adnexal inflammatory changes according to the laparoscopic criteria mentioned in the text

† Day 0 represents the day of laparoscopy

of IgG and IgA just above the upper limit for the normal range (1680 and 315 mg per 100 ml) were found in 11 and 3 of the 53 cases respectively, while the other patients had concentrations of IgG and IgA within the normal range.

In 14 of the 18 patients with high levels of IgM an increased concentration of this immunoglobulin class was found already in the sera collected on the day the patients were admitted to the hospital (Table 1). Of the cases presented in Table 1, 10 reported a debut of abdominal pain less than 1 week before hospitalization. In five of the patients this symptom had been present for 1 to 2 weeks and in three for more than 3 weeks. No correlation was found between the serum level of IgM and the time that had passed since the debut of abdominal pain. Nor could any correlation be demonstrated between the severity of the adnexal inflammatory changes and the serum concentration of IgM. Six of the salpingitis patients with increased levels of IgM had inflammatory changes in the adnexa which were considered as stage A, 10 as stage B and two as stage C. The corresponding figures for the 35 cases that had IgM levels within the normal range were 11, 14 and 10.

Five of the 18 cases with increased levels of IgM had a concentration of IgG just above the upper limit for the normal range (Table 1). In some cases (No. 4, 6, 7 and 8) an increase of the concentration of IgG was seen during the period of observation. Moderately increased levels of IgG were found in six of the 35 cases that had serum concentrations of IgM within the normal range. IgA levels just above the upper limit for the normal range were observed in two of the salpingitis patients with high concentrations of IgM, whereas in one of these latter cases (No. 7) there was a more pronounced increase of IgA (Table 1). IgA was within the normal range in all the 35 patients with normal concentrations of IgM.

Immunoglobulin Levels in Women with Infections Confined to the Lower Genital Tract and in Healthy Women

The concentrations of IgM in the sera from the 20 patients with infections confined to the lower genital tract (Group II) were in all except two instances within the normal range. The concentrations of IgM in these two patients were 260 and 215 mg per 100 ml. IgG and IgA were within the normal range in all patients in this group.

The serum levels of IgG, IgA and IgM in the 20 healthy females (Group III) were, except in one woman, within the normal range. In this woman the concentrations of IgG, IgA and IgM were 1227, 154 and 235 mg per 100 ml. One woman in Group II and the one in Group III who had increased levels of IgM had earlier been treated for salpingitis.

Increase of IgM in Relation to Infections by M. hominis

The number of females within the three investigated groups from whom *M. hominis* was isolated and who had indirect haemagglutinating (IHA) antibody to *M. hominis* at a titre of 1:16 or greater is shown in Table 2.

Cultures for *M. hominis* were made from 15 of the 18 salpingitis patients with increased levels of IgM. *M. hominis* was isolated from the cervix in 14 of these patients and in pure cultures from the Fallopian tubes in two. With the exception of *M. hominis* no other micro-organisms were isolated from the Fallopian tubes in those cases that had increased serum levels of IgM. IHA antibody to *M. hominis* at a titre of 1:16 or greater was found in 17 of these 18 patients (Table 2). IHA antibody at a titre 1:16 or greater was found significantly more often in those salpingitis cases that had increased levels of IgM than in those with concentrations of IgM within the normal range ($p < 0.001$). A significant rise in the titre of antibody to *M. hominis* was demonstrated in six of the 35 salpingitis cases from which

TABLE 2 *Isolation Rate of M hominis and Incidence of IHA Antibody to M hominis at a Titre of 1 16 or Greater within the Different Groups of Investigated Females*

Group	No of cases	Concentration of IgM in serum	n	*M hominis* isolated	IHA antibody to *M hominis*	Significant rise of the titre of IHA antibody[a]
I	53	increased[b]	18	14[d]	17	5(12)
		normal[c]	35	21	10	1(23)
II	20	increased	2	2	-	0(1)
		normal	18	8	6	0(9)
III	20	increased	1	1	1	0(1)
		normal	19	0	1	0(9)

[a] *The number of cases from which consecutive serum specimens were collected is shown within parentheses*

[b] > 155 *mg per 100 ml*

[c] 25 to 155 mg per 100 ml

[d] Cultures were performed from 15 of the 18 cases

[e] Cultures were performed from 31 of the 35 cases

consecutive serum specimens were collected Of these six cases five (case No 1 2, 4 7 and 10) had increased levels of IgM (Table 1)

The patients in Group II and III who had increased levels of IgM harboured *M hominis* in the lower genital tract and had IHA antibody to this organism (Table 2)

Repeated cultures were made from the cervix and the urethra of the salpingitis patients during the course of the disease These cultures showed that the treatment (penicillin in combination with chloramphenicol) given to the majority of the salpingitis patients did not have any influence on the occurrence of *M hominis* in the lower genital tract

Neisseria gonorrhoeae was isolated from the lower genital tract but not from the uterine tubes in three of the salpingitis cases with increased levels of IgM These three women also harboured *M hominis* From the salpingitis patients with IgM levels within the normal range gonococci were recovered from the cervix in 12 instances and in pure culture from the Fallopian tube in two From four patients in Group II …nococci were isolated from the lower genital tract None of them had increased leve… of IgM

The sera from those individuals who had increased serum levels of IgM were tested in Wassermann and Meinicke reactions which in all instances were negative Complement fixation antibody to *M pneumoniae* at a titre greater than 1 10 or cold agglutinins at a titre greater than 1 16 were not found in any of these sera

DISCUSSION

A marked increase of the serum level of IgM in combination with moderately increased levels of IgG and IgA have been reported in patients with bartonellosis (*Heremans* 1963) infectious mononucleosis (*Wollheim & Williams* 1966) malaria (*Tobie et al* 1966 infectious hepatitis (*Wollheim* 1968) and with mumps and brucellosis (*Zanussi & Medina* 1968 A selective increase of IgM has been demonstrated in *M pneumoniae* infections ([illegible] 1967 *Oxelius* 1969) and in trypanosomiasis (*Mattern et al* 1961)

High concentrations of IgM with concomitant normal concentrations of IgG and IgA have also been observed in late syphilis (*Laurell et al* 1968) The clinical picture as well as negative Wassermann and Mei

nicke reactions contradict that the increase of IgM in the salpingitis patients was caused by a syphilitic infection

An immunoglobulin pattern similar to that observed in the salpingitis cases has also been found in patients with *M pneumoniae* infections (*Feiz* 1967) The salpingitis patients had no signs of respiratory tract infections In none of the cases with an increased level of IgM was the result of the complement fixation test with *M pneumoniae* suggestive of an infection by this organism Furthermore cold agglutinins were not found in any of the sera with increased concentrations of IgM

The present study does not indicate that uncomplicated gonorrhoea causes a marked increase of the serum level of IgM IgM was also within the normal range in those salpingitis cases from whom gonococci were isolated from the Fallopian tubes Of the 13 salpingitis patients from whom gonococci were recovered from the lower genital tract but not from the uterine tubes three had however increased levels of IgM Cervical cultures in these three cases also yielded growth of *M hominis*

The increase of IgM in patients with acute salpingitis may partly be explained by a synthesis of antibodies of IgM class formed against the micro organism causing the infection With the exception of *M hominis* no other micro organisms were isolated from the Fallopian tubes in those salpingitis cases that had increased serum levels of IgM *M hominis* was recovered from the cervix in all except one and IHA antibody to this organism was detected in 17 of the 18 cases with high concentrations of IgM No correlation could however be demonstrated between the serum level of IgM and the titre of IHA antibody to *M hominis* In most of the infectious diseases causing a marked increase of IgM it has not been possible to demonstrate any correlation between the serum level of IgM and the titre of a certain antibody formed against the infectious agent (*Zanussi* & *Medina* 1968)

The serum concentration of IgM in healthy young adult females is generally higher than in males in the corresponding ages (*Johansson et al* 1968) In the group of healthy females in the present series the mean value of the serum concentration of IgM was 89 mg per 100 ml and the range 40 to 142 mg per 100 ml (excluding the case with 235 mg per 100 ml which had a history of earlier salpingitis) The corresponding figures for the standard serum pool which comprised samples from mainly males of the same ages as the females were for the mean value 77 mg per 100 ml and for the range 25 to 155 mg per 100 ml Similar values for the serum concentration of IgM in healthy young adult females and males were found by the above mentioned authors The 18 salpingitis patients presented in Table 1 had in one or more samples a serum level of IgM which was about two and a half to four times higher than the mean value of the concentration of IgM in the group of healthy females

Antigenic stimulation generally causes a transient early synthesis of antibodies of IgM class subsequently followed by a synthesis of antibodies preferentially of IgG class The nature of the antigen can however influence the kinetics of the antibody response (*Torrigiani* & *Roitt* 1965) A long lasting 19 S antibody response has been demonstrated in patients with *M pneumoniae* infections (*Biberfeld* 1968) Experimental studies have also shown that the transient IgM antibody response may be maintained by repeated administration of antigen (*Torrigiani* & *Roitt* 1965) Therefore the continuation of the IgM antibody response may be related to the persistence of the infectious agent in the body In one of the salpingitis patients in the present series an increased serum level of IgM was found in a specimen collected about 6 months after the acute stage of the disease This patient still harboured *M hominis* Repeated cultures made from the cervix of the salpingitis patients during the course of the disease showed that the antibiotic treatment (penicillin in combination with chloramphenicol) given to the patients did not influence the presence of *M hominis*

An adequate antibiotic treatment as to *M hominis* might have caused a more rapid decrease of the serum concentration of IgM

Another explanation of the increase of IgM in women with acute salpingitis might be that it partly represents antibodies of the IgM class formed against tissue antigens The peculiar habit of mycoplasma organisms to localize at the surface of infected cells may represent a stimulus for sensitization not only to the micro organisms but also to elements of the cells themselves (*Thomas* 1969) Antibodies to tissue antigens have been demonstrated in the sera of patients with *M pneumoniae* infections (*Thomas* 1943 *Biberfeld* 1970) *Biberfeld* (1970) found such antibodies to be exclusively of IgM class

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A ROLE FOR RED CELLS IN PHYTOHAEMAGGLUTININ-INDUCED LYMPHOCYTE STIMULATION

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The phytohaemagglutinin induced lymphocyte stimulation was studied in the presence of various types of blood cells The response of purified lymphocytes to PHA was reduced compared with the response of lymphocytes from buffy coat of whole blood When red cells were added to the purified lymphocytes the response was restored Leucocytes also had some restoring effect

Phytohaemagglutinin (PHA) induces morphological (*Nowell* 1960 *Yoffey et al* 1965) and physiological (*Cooper et al* 1963) changes in peripheral lymphocytes *in vitro* and renders the lymphocytes cytotoxic (*Holm & Perlmann* 1969) Similar changes in lymphocytes are induced by antigens against which the lymphocyte donor is sensitized (*Elves et al* 1963)

A significant role has been ascribed to macrophages in lymphocyte stimulation by antigens (*Roitt et al* 1969) On the other hand no decisive role of macrophages in PHA induced lymphocyte stimulation has been found The removal of phagocytes from the lymphocyte preparation has been reported both to decrease (*Wilson* 1966) and to increase (*Walker & Fowler* 1965) the response of the lymphocytes to PHA The response to suboptimal doses of PHA has been found to be reduced by removal of phagocytes whereas the response to optimal doses was unaffected (*Oppenheim et al* 1968)

In the present investigation the influence of various blood cells on PHA induced lymphocyte stimulation was studied The lymphocyte response was low when few other blood cells were present in the reaction mixture Some increase was obtained by the addition of leucocytes Surprisingly the addition of red blood cells (RBC) caused a response as high as that of lymphocytes in buffy coat of whole blood

MATERIALS

Materials were obtained from the following sources Heparin from Vitrum AB Stockholm Sweden benzylpenicillin from KABI AB Stockholm Sweden streptomycin sulphate from Glaxo Laboratories Ltd Greenford England [^{3}H methyl] thymidine (specific activity 6 7 Ci per m mole) from New England Nuclear Corp Boston Massachusetts water soluble papain and cetyltrimethyl ammonium bromide (CTAB) from Merck AG Darmstadt Germany red kidney beans (*Phaseolus vulgaris*) Weibull no 44 from Weibullsholm Plant Breeding Institute Landskrona Sweden sterile disposable nylon fibre filters Leukopak® from Fenwall Laboratories Morton Grove Illinois

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a synthetic medium TCM 199 from Flow Laboratories Irvine Ayrshire Scotland Mitomycin C from Sigma Co St Louis Missouri

PHA was purified as described by *Borjeson et al* (1964) omitting the chromatography step Aliquots of the same PHA preparation stored at —20 C were used throughout the investigation

METHODS

Preparation of lymphocytes Venous blood was obtained from healthy donors Each sample of 400 ml was collected into a sterile bottle containing 6000 IU of heparin in 4 ml saline Lymphocytes were isolated by nylon fibre filtration and differential centrifugation in colloidal silica polyvinylpyrrolidone as previously described (*Tarnvik* 1970)

Culture medium The culture medium consisted of 80 per cent TCM 199 and 20 per cent pooled inactivated (56 C 30 min) human serum Penicillin and streptomycin were added to the final concentrations of 150 units and 150 µg per ml respectively Thirty µg of PHA in 50 µl deionized water were added per ml of lymphocyte suspension

Lymphocyte culture Lymphocytes were suspended to 0 7 × 10^6 cells per ml in culture medium and 1 5 ml volumes of the suspension were distributed in 16 × 110 mm round-ended glass tubes In most experiments 0 2 ml of a blood cell suspension was added to each tube The tubes were incubated at 37 C for 6? hours in a 30 × 28 × 22 cm closed humidified plexiglass box. The box was flushed each day for 45 min with 5 litres of air supplemented with 5 per cent CO_2 (v/v)

Blood cell suspensions added to lymphocyte cultures Various types of cells were obtained from the same blood sample as the lymphocytes (Table 1) Homologous and sheep RBC obtained from ous blood were washed in TCM 199 supplemented with 2 per cent pooled heat inactivated human serum After washing the cells were suspended in culture medium

Measurement of the incorporation of ³H thymidine into DNA Fourteen hours before the end of the incubation period 1 5 µCi of H thymidine in 30 µl deionized water was added to each tube After incubation 0 2 ml of a suspension of sheep RBC diluted with 99 parts of 0 16 M NaCl was added to each tube in order to visualize the cell button during the washing procedure The cells were washed three times in 5 ml 0 9 per cent (w/v) saline with centrifugation at 1700 rpm 500 × g) The tubes were stored for 1 day to 3 weeks at —20 C before the determination of radioactivity incorporated into DNA The cell materials were then digested with papain and nucleic acids were precipitated with CTAB (*Borjeson et al* 1966) The precipitate was dissolved in 0 5 ml of a mixture of n propanol and deionized water (1 1) and added to 10 ml of liquid scintillation solution (*Bray* 1960) The radioactivity was estimated in a liquid scintillation spectrometer

Morphology of cells At the end of the incubation period the cells were harvested by centrifugation at 950 rpm (160 × g) for 5 min The supernatant fluid was sucked off one drop of human pooled inactivated (56 C 30 min) serum was added and Giemsa stained smears were prepared from which 400 cells were counted

Mitomycin treatment To prevent DNA synthesis the cells were treated with mitomycin C as described by *Bach & Voynow* (1966) The treatment was checked to be effective in so far as the incorporation of ³H thymidine into DNA in PHA cultures was insignificant

RESULTS

PHA response of various lymphocyte preparations The PHA response of lymphocytes in various preparations is shown in Table 2 Lymphocyte density was kept constant in the preparations Lymphocytes from buffy coat of whole blood and buffy coat of filtered blood incorporated ³H thymidine to about the same extent Thus removal of phagocytes did not affect the PHA response The purified lymphocyte suspension virtually depleted of other leucocytes and RBC showed low incorporation of ³H thymidine into DNA when compared with lymphocytes from buffy

TABLE 1 *Sources of Autologous Blood Cells Added to Lymphocyte Suspensions (A Tarnvik 1970)*

A	Cells from whole blood
B	Cells from filtered blood i e whole blood with a reduced number of phagocytic cells
C	Cells from buffy coat of whole blood
D	Cells from the mononuclear band collected after centrifugation of non filtered blood cells This band contained monocytes lymphocytes and platelets in about the same relative proportions as whole blood Few RBC and polymorphonuclear cells were present
E	Cells from the polymorphonuclear band collected after centrifugation of non filtered blood cells This band contained polymorphonuclear cells and RBC in about the same numbers Few mononuclear cells were present
F	Purified lymphocytes

TABLE 2 *The Incorporation of ³H Thymidine into DNA by Lymphocytes Prepared in Various Ways from the Same Blood Sample The Lymphocytes were Incubated with PHA*

Lymphocyte preparation	Counts × 10³ per min Mean ± S D of 5 cultures
Buffy coat of whole blood	85 7 ± 2 0
Buffy coat of filtered blood	81 0 ± 6 2
Purified lymphocytes	37 3 ± 10 7
Purified lymphocytes with added filtered blood cells (4 × 10⁶ RBC per ml finally)	91 2 ± 8 9

coat When 4 × 10⁶ RBC in filtered blood (B in Table 1) were added per ml of purified lymphocyte suspension the incorporation was of the same order of magnitude as in lymphocytes from buffy coat

Influence of various RBC preparations on the lymphocyte response to PHA Purified lymphocytes were incubated in the presence of various numbers of washed RBC from

TABLE 3 *Effect of RBC on the Response of Purified Lymphocytes to PHA as Measured by Incorporation of Radioactivity and Formation of Blast Like Cells Means ± S D of 5 Cultures are Indicated*

Cells added	Counts × 10³ per min	Per cent blast like cells
10 RBC per ml	98 1 ± 12 9	40 ± 20
No cells	20 2 ± 4 5	4 ± 1

TABLE 4 *Effect of Autologous Homologous and Sheep RBC on the Response of Purified Human Lymphocytes to PHA The Incorporation of ³H Thymidine into DNA was Measured*

RBC added (10⁷ cells per ml)	Mean counts per min ± S D of 5 cultures	
	PHA present	PHA not present
Autologous	37 035 ± 7 218	77 ± 23
Homologous	45 073 ± 9 245	117 ± 18
Sheep	23 929 ± 6 392	78 ± 26
No cells	8 120 ± 1 277	72 ± 4

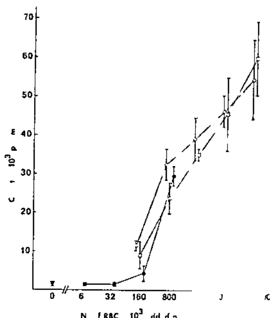


Fig 1 Effect of RBC on the lym[illegible] to PHA The incorporation of [illegible] DNA was measured in PHA lym[illegible] containing different numbers of R[illegible] and standard deviations of 5 cultu[illegible] The RBC were added in the forms [illegible] washed cells from whole blood (○) [illegible] blood (□) and from buffy coat of w[illegible] (●) The composition of these three cel[illegible] pensions were

Cell source	Polymorphonuclear cells + monocytes/10³ RBC	Lymphocytes/10³ RBC	Platelets/10³ RBC
○ Whole blood	430	460	38 000
□ Filtered blood	3	450	380
● Buffy coat of whole blood	150 000	140 000	19 × 10

whole blood from filtered blood and from buffy coat of whole blood The proportions of various leucocytes and platelets in relation to the numbers of RBC varied widely (Table in the legend to Fig 1) The incorporation of H thymidine into DNA was correlated to the number of RBC added (Fig 1)

TABLE 5 *Effect of Various Cells on Lymphocyte Response to PHA The Incorporation of ^{3}H Thymidine into DNA was Measured The Added Cells were Mitomycin Treated*

They were

a) Cells from the mononuclear band obtained after centrifugation of autologous non filtered blood cells i e monocytes lymphocytes and platelets in about the same relative proportions as in whole blood

Cells added	Counts × 10^{3} per min Mean ± S D of 5 cultures
10^{6} mononuclear leucocytes	5.8 ± 0.5
10^{6} RBC	10.7 ± 1.8
No cells	0.7 ± 0.1

b) Cells from the polymorphonuclear band obtained after centrifugation of autologous non filtered blood cells i e polymorphonuclear leucocytes and RBC in about the same numbers

Cells added	Counts × 10^{3} per min Mean ± S D of 5 cultures
10 polymorphonuclear leucocytes + 10 RBC	16.4 ± 0.6
10^{5} RBC	10.9 ± 1.1
No cells	4.0 ± 0.3

c) Purified lymphocytes

Cells added	Counts × 10^{3} per min Mean ± S D of 5 cultures
4 × 10^{6} lymphocytes	[illegible].9 ± 0.[illegible]
4 × 10^{6} RBC	72.2 ± 9.3
No cells	2.3 ± 0.2

Morphological examination showed that the increase in radioactivity reflected the increase in frequency of blast like cells (Table 3)

In three experiments mitomycin treated RBC from filtered blood (B in Table 1) were added to purified lymphocytes They had the same effect on the incorporation of ^{3}H thymidine into DNA as did non treated cells

Homologous RBC and sheep RBC influenced the incorporation of ^{3}H thymidine into DNA to about the same extent as autologous RBC These cells caused no stimulation in the absence of PHA (Table 4)

Influence of various autologous leucocyte platelet preparations on the lymphocyte response to PHA A preparation containing mitomycin treated monocytes lymphocytes, and platelets (D in Table 1) caused higher incorporation of ^{3}H thymidine into DNA of purified lymphocytes (5 800 cpm) than the incorporation obtained when no cells were added (700 cpm) RBC in the same number as the mononuclear cells of this preparation caused a still higher incorporation (10 700 cpm) (Table 5 a)

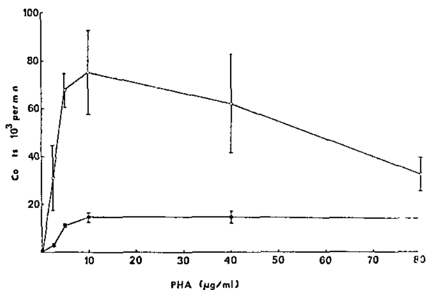
Evaluation of the influence of polymorphonuclear cells (E in Table 1) on the response of purified lymphocytes to PHA was difficult due to the presence of RBC in the added preparation Polymorphonuclear cells appeared to cause an increase in the response (Table 5 b)

Mitomycin treated purified lymphocytes (F in Table 1) did not affect the incorporation of ^{3}H thymidine into DNA to any appreciable extent (Table 5 c)

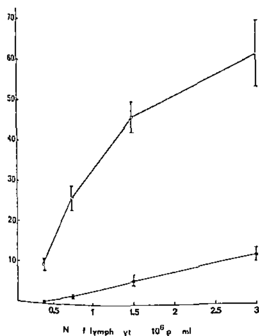
Influence of autologous plasma on the lymphocyte response to PHA In two experiments 0.2 ml of autologous plasma was added to each of 5 lymphocyte cultures No influence on the incorporation of ^{3}H thymidine into DNA was found

Influence of RBC on the lymphocyte response to PHA with varying PHA dose cell density incubation period and period of exposure to RBC Purified lymphocytes were incubated with 2.5 to 80 µg of PHA per ml When RBC were present an optimal incorporation was found at 5 to 40 µg of PHA per ml and a decrease at 80 µg per ml When lymphocytes were incubated without RBC the response was significantly lower over the whole dose range tested and no decrease at the high dose was found (Fig 2 a)

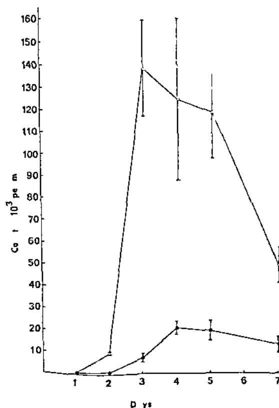
When the density of purified lymphocytes



a



b



c

Fig 2 Effect of RBC o the response of purified l mphocytes to PHA w th varying
a) PHA dose
b) cell den ity
c) incubation per od
○——○ 10^7 autologous RBC added per ml
●——● No RBC added
The incorporation of H thymidine into DNA as m asured Means ± S D of 5 cultures are ind cated

was varied from 0 375 × 10⁶ to 3 × 10⁶ cells per ml in the presence of a constant number of RBC (B in Table 1) the incorporation of ³H thymidine into DNA was invariably higher than in cultures without RBC (Fig 2 b)

Purified lymphocytes were incubated for 1 to 7 days, and ³H thymidine was present for the last 14 hours of incubation In all periods higher incorporation of ³H thymidine into DNA was found when RBC (B in Table 1) were present The highest incorporation was found after 3 to 5 days of incubation (Fig 2 c)

Purified lymphocytes were incubated for 62 hours and RBC (B in Table 1) were added at various intervals *before the end of* the incubation period The highest incorporation was found when RBC were present from the beginning When RBC were added 14 hours before the end of incubation they had no influence on the incorporation (Fig 3)

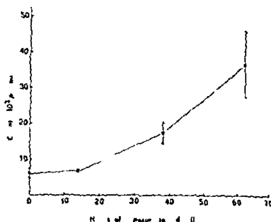


Fig 3 Effect of RBC on the response of purified lymphocytes to PHA with varying periods of exposure to RBC Lymphocytes were incubated for 62 hours and RBC were added 0 14, 38 and 62 hours before the end of incubation The incorporation of ³H thymidine into DNA was measured Means ± S D of 5 cultures are indicated

DISCUSSION

Studies on the possible interaction between cells in PHA induced lymphocyte stimulation have mainly dealt with the influence of phagocytes *Wilson* (1966) and *Pertoft et al* (1968) in contrast to *Walker & Fowler* (1965) *Ciresa & Huber* (1967) and *Schellekens & Eijsvoogel* (1968) found a lower PHA response when lymphocytes were purified from phagocytes than in phagocyte rich preparations The present results indicate that a possible influence of phagocytes on the PHA response may be masked if great numbers of RBC are present in the cultures About the same degree of response was obtained in two lymphocyte preparations rich in RBC one of which was low in phagocytes Moreover, when various types of RBC preparations were added to purified lymphocytes there was no correlation between the degree of response and the number of phagocytes present However when a preparation containing monocytes lymphocytes and platelets and virtually free from RBC, was added to purified lymphocytes, a higher response was found than in a culture of purified lymphocytes without added cells. Polymorphonuclear cells appeared to affect the response in the same way but the results were difficult to evaluate as RBC occurred in about the same number as the polymorphonuclear cells It is possible that the discrepancy between earlier reports on the influence of phagocytes on PHA action is due to the variation in the numbers of RBC present in the cultures Thus *Pertoft et al* (1968) preparing lymphocytes free from RBC, found a higher PHA response with than without phagocytes present in the cultures The other reports are not easily evaluated in this respect as the numbers of RBC are not stated

An influence of RBC on lymphocyte stimulation by PHA has been noticed previously *Carstairs* (1962) wrote that the presence of some red cells seems to benefit the culture *Allen et al* (1969) separated a PHA preparation into three lymphocyte stimulating proteins one of which was strongly haemagglutinating They reported in passing that the lymphocyte response to this protein was

potentiated by RBC when the protein was used in high doses In the present study, performed independently of that of *Allen et al* (1969) a pronounced effect of RBC was found over a wide range of PHA doses *Johnson & Kirkpatrick* (1970) have recently reported that RBC and RBC membranes augment the PHA induced DNA synthesis of lymphocytes When the RBC lymphocyte ratio was increased from 1 to 100 the radioactivity recorded increased from 310 000 to 520 000 dpm In the present study the lowest RBC lymphocyte ratio tested was < 0 01, which is probably why the influence of RBC was so strikingly demonstrated (Fig 1) However the influence of RBC on PHA action may vary widely when various PHA preparations are used It must also be emphasized that cells from the thoracic duct mainly lymphocytes, have been found to respond well to PHA (*Cooper et al* 1963 *Lindahl Kiessling & Werner* 1964) The presence of cells other than the lymphocytes is thus not a prerequisite for PHA stimulation

Thomson et al (1966) have studied the PHA response of lymphocytes freed from phagocytes by filtration and from RBC by hypotonic shock treatment The response was lower after hypotonic treatment than before the treatment which was suggested to indicate the existence of two or more lymphocyte populations one of which was more fragile and more responsive to PHA In the light of the report of *Allen et al* (1969) and the present results it is possible that the low response of lymphocytes after hypotonic shock treatment was due to the loss of RBC

When RBC were present in the reaction mixture only for the last 14 hours of the incubation period they did not influence the response to PHA If RBC served a nutritive function in the present culture model they would be expected to be important even at the end of the culture period when metabolic activity is high The possibility of a nutritive function for the RBC is also contradicted by results suggesting that RBC do not influence the lymphocyte response to allogenic cells (*Tarnvik* to be published)

The PHA dose response curve obtained when RBC were present agrees with earlier results (*Mueller & Mahieu* 1966 *Forsdyke* 1967 *Rigas & Tisdale* 1969) *i e* the response increased rapidly with the dose at low doses and decreased slowly with the dose at high doses The decrease at high doses was not found in the present investigation when purified lymphocytes were cultured in the absence of RBC This observation suggests that the decrease in PHA response at high doses is not necessarily due to a toxic effect of PHA on the lymphocytes The high PHA doses cause strong haemagglutination Experiments in progress indicate that the role of RBC in PHA action may be played by red cell membranes which is in agreement with the report by *Johnson & Kirkpatrick* (1970) The reduced response at high PHA dose may thus at least partly be due to a reduction in the total available membrane surface caused by agglutination

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PROTEOLYTIC ENZYMES AND BIOLOGICAL INHIBITORS

III Naturally Occurring Inhibitors in some Animal and Plant Materials and their Effect upon Proteolytic Enzymes of Various Origin

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The Veterinary College of Norway Oslo Norway
Head Professor dr med vet Olav Sandvik

It was demonstrated with the aid of the casein precipitating inhibition test (CPI test) that chicken egg white colostrum from different animal species and extracts of grain and potato contained inhibitory substances against trypsin α-chymotrypsin a number of microbial proteinases and ficin and papain Extracts of *Ascaris suum* contained inhibitory substances which were active against the animal and several of the microbial proteinases tested but not against ficin and papain A semiquantitative measure for the inhibitory capacity of chicken egg white different colostrum species extracts of wheat barley rye and corn flours and extracts of potato upon various enzymes is given By combination of paper electrophoresis of the inhibitor containing materials with the CPI test (electrophoretic CPI test) it was found that each of the materials investigated contained several electrophoretically distinguishable inhibitors with different inhibitory spectra Some of these inhibitors were active against one single enzyme of animal or microbial origin others seemed to be active against two enzymes such as trypsin and α-chymotrypsin proteinases from *Bacillus subtilis* and *Aspergillus oryzae* or ficin and papain while still others affected a variety of different enzymes Some inhibitors seemed to affect swine trypsin but not bovine trypsin Of the two enzyme fractions in the crude *Aspergillus oryzae* proteinase only one was affected by many of the inhibitory substances The number of inhibitors as well as the pattern of the different inhibitors in colostrum from different individuals of the same species seemed also to differ Many of the inhibitors demonstrated have not been reported previously

Naturally occurring inhibitors of trypsin have been found in several materials of animal and plant origin as reviewed by *Vogel et al* (1968) Although the effect of the inhibitors upon trypsin and in many cases chymotrypsin has been most thoroughly studied other proteolytic enzymes are also frequently affected

In a previous paper *Fossum* (1970) reported a close correlation between the casein precipitating inhibition test (CPI test) and the Kunitz method when testing the interaction between some commercial enzymes and inhibitors The CPI test was considered to be of value especially when test

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ing the interaction of enzymes and inhibitors of low activities, and when screening the effect of many different inhibitors upon various enzymes By electrophoresis of various animal sera combined with the CPI test (electrophoretic CPI test) *Fossum* (in press) showed that all the sera tested contained two or more proteolytic enzyme inhibitors, with different inhibitory spectra

The aim of the present work was to study, by use of the electrophoretic CPI test the inhibitory spectra and the complexity of the inhibitors, in some animal and plant materials upon proteolytic enzymes of various origin

MATERIALS AND METHODS

Enzymes

The following enzymes were obtained from Sigma[1] Trypsin from bovine pancreas (Type III 2 × crystallized lot 97B 8000) α-chymotrypsin from bovine pancreas (Type II 3 × crystallized lot 86B-0470) protease from *Bacillus subtilis* (Type VIII crystallized lot 17B 2770) protease from *Aspergillus oryzae* (Type II crude lot 1320) ficin from fig tree latex (2 crystallized lot 119B-4750) papain from papaya latex (2 × crystallized lot 47B-1020) Trypsin from hog pancreas (crystallized batch No 36467) was obtained from Koch Light Laboratories Ltd[2] The other microbial enzymes used were generally prepared as described previously *Fossum* in press) The crude commercial proteinase from *Aspergillus oryzae* was chromatographed on a DEAE cellulose column using acetate buffer pH 5 6 with a gradient from 0 005 M to 0 05 M containing 2 per cent sodium chloride as eluent In this way two distinct peaks referred to as Fraction I and II were obtained

The enzyme solutions were usually stored at +4 C with the addition of merthiolate to a final concentration of 1 10 000 The strength of the enzyme solutions used in the CPI test was usually of the order of 100 to 1000 CP units per 0 025 ml (*Fossum* 1970) In the case of ficin and papain sulfhydryl activators were added to the enzyme solutions

Inhibitor Containing Materials

Ovomucoid trypsin inhibitor was obtained from Sigma (chicken egg white type II-0 lot 27B-8500) and from Koch Light (5554t) Chicken egg white was taken from commercially available eggs Milk from the various animal species tested was taken within 24 hours after parturition Human milk was from the first day of lactation (third day after parturition) The milk samples were centrifuged the skim milk collected and used for the tests without further concentration or purification *Ascaris suum* was collected from intestines of autopsied pigs The whole worms were washed thoroughly in saline then homogenized in saline centrifuged and the clear supernatant used Commercial flour of barley rye and wheat were suspended in 10 × the weight of saline the suspensions were allowed to stand for one hour and then centrifuged Ammonium sulphate was added to the supernatants to a final concentration of 80 per cent saturation at +4 C the precipitates were collected and then dialysed Similar extracts of *corn* (maize) were made by homogenization of the grain collected from commercially available material Extracts of potatoes were made by grating the potatoes adding an equal amount of saline and treating the liquid phase as described above

The inhibitor-containing preparations were preserved by the addition of merthiolate to a final concentration of 1 10 000 and stored at −20 or +5 C until used Two or more different batches of each of the inhibitor containing material were usually examined

Determination of Inhibitory Activity

The CPI test was performed as described by *Fossum* (1970 in press) using as substrate sodium caseinate incorporated into agar Electrophoretic differentiation of the inhibitors in the different materials was carried out in the same way as for the serum inhibitors by use of an electrophoresis apparatus type 3276BN LKB[3] (*Fossum* in press)

In order to obtain the electrophoretic patterns of the various inhibitory substances different dilutions of the inhibitor-containing materials were subjected to electrophoresis after which developing was performed with different dilutions of the enzymes The inhibitory spectra and the number of inhibitors indicated on the figures are in each case based upon a series of experiments and not only upon the data obtained from the particular figures shown

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[1] Sigma Chemical Company St L. Mo USA

[2] Koch Light Laboratories Ltd Colnb k Buckinghampshire England

[3] LKB-Produkter P O box 12035 Stockholm 12 Sweden

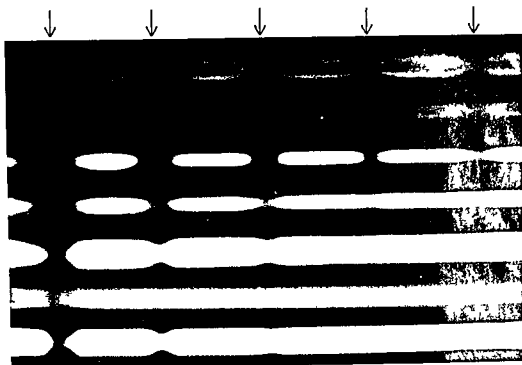


Fig 1 Crosswise CPI test with different dilutions of chicken egg white The dilutions are (from left to right as marked by arrows) 1 2 1 10 1 50 1 200 and 1 500 Enzymes are (downwards) Swine trypsin (0 005 mg per ml) α chymotrypsin (0 005 mg per ml) proteinases from *Bacillus subtilis* (0 001 mg per ml) *Aspergillus oryzae* (0 03 mg per ml) *Bacillus cereus Staphylococcus aureus* and *Corynebacterium pyogenes*

RESULTS

Egg White Inhibitors

Fig 1 shows the inhibitory activity of different dilutions of egg white upon different proteolytic enzymes It can be seen that some of the enzymes are inhibited by a dilution of the egg white of 1 500 while others are inhibited only to a very small extent Bacterial proteinases not presented in the figure such as those of *Proteus vulgaris Pseudomonas aeruginosa* and *Serratia marcescens* were inhibited to approximately the same extent as the *Bacillus cereus* proteinase The same was true for ficin and papain Fig 2 shows the electrophoretic patterns of the inhibitors in chicken egg white against different proteolytic enzymes Five electrophoretically distinguishable inhibitors with different inhibitory spectra are demonstrated Inhibitor I giving rise to the partial inhibition of swine trypsin could not be shown to inhibit bovine trypsin nor any of the other enzymes tested Inhibitor II causes inhibition of both bovine and swine trypsin but does not inhibit the other enzymes Inhibitor III inhibits α chymotrypsin all the microbial enzymes tested including those of *Proteus vulgaris Pseudomonas aeruginosa* and *Serratia marcescens* and also the plant proteinases ficin and papain Electrophoresis of diluted egg white and developing with different enzyme dilutions indicated that this inhibitor does not inhibit trypsin Ficin and papain are also inhibited by another anodic migrating inhibitor (IV) Further an inhibitor (V) on the cathode side of the line of application affects trypsin α chymotrypsin *Bacillus subtilis* proteinase and Fraction I from *Aspergillus oryzae* proteinase while Fraction II as well as the other bacterial proteinases tested were not inhibited in this area

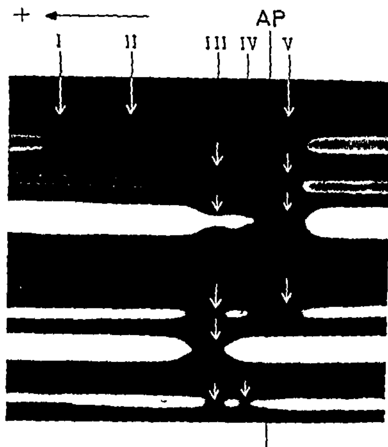


Fig 2 Electrophoretic CPI test of chicken egg white The enzymes used are (downwards) Swine trypsin α-chymotrypsin proteinases from *Bacillus subtilis Aspergillus oryzae* and *Bacillus cereus* in the same concentrations as in Fig 1 and ficin (0 05 *mg per ml*) The electroohoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 hours The electrophoretically distinguishable inhibitors resulting in zones of inhibition are given numbers from left to right and the zones of inhibition are marked by arrows

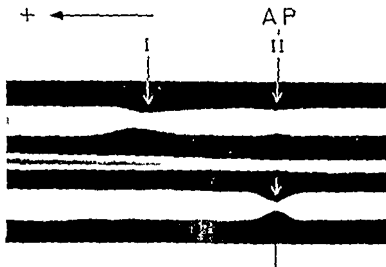


Fig 3 Electrophoretic CPI test of ovomucoid trypsin inhibitor The enzymes used are (downwards) Swine trypsin α-chymotrypsin and proteinase from *Bacillus subtilis* The strength of the enzymes used and other conditions are the same as in Figs 1 and 2

Fig 3 shows that electrophoresis of crystallized ovomucoid trypsin inhibitor results in two zones of inhibition (I and II) for trypsin, one of which (II) corresponds to a zone of inhibition for *Bacillus subtilis* proteinase

Colostrum Inhibitors

Table 1 presents the inhibitory activity of one colostrum sample from each of the species tested upon different proteolytic enzymes Bovine and swine trypsins are inhibited to the same extent and in most cases

TABLE 1 *Highest Dilution of Colostrum from Various Species Resulting in Inhibition of Different Proteolytic Enzymes The Data are Based upon the Crosswise CPI Test*

Proteolytic enzymes (Origin)	Colostrum species						
	Human	Pig	Cattle	Sheep	Goat	Horse	Dog
Trypsin (bovine and swine)	1 50*	1 1000	1 100	1 100	1 100	1 200	1 200
α Chymotrypsin	1 50	1 500	1 50	1 50	1 50	1 100	1 500
Bacillus subtilis	1 50	1 100	1 50	1 10	1 50	1 100	1 1
Aspergulus oryzae	1 1	1 100	1 10	1 10	1 50	1 100	1 1
Bacillus cereus	0†	1 100	1 10	1 10	1 10	1 50	1 50
Corynebacterium pyogenes	0	1 500	1 100	1 10	1 100	1 1	0
Staphylococcus aureus	0	1 50	1 50	1 50	1 10	1 1	1 1
Proteus vulgaris	0	1 10	1 1	1 1	0	1 1	1 1
Pseudomonas aeruginosa	0	1 100	1 50	1 10	1 10	1 10	1 10
Serratia marcescens	0	1 50	1 10	1 1	1 1	1 1	1 1
Ficin and papain	1 10	1 10	1 10	1 1	1 1	1 10	1 10

* Each colostrum species was diluted 1 1 1 10 1 50 1 100 1 200 1 500 1 1000 and 1 2000 in saline

† 0 No inhibition observed

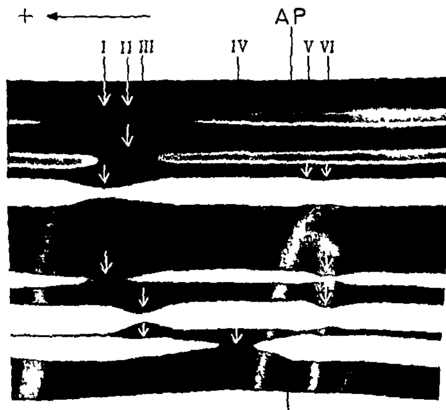


Fig 4 Ele ophoret c CPI test of bo ine colostrum The enzymes used are (downwards) sin α-chymotrypsin proteinases from *Bacillus s btiis Asperg llus o y ae Bacillus cereu* *bacterium pyogenes* The strength of the enzymes used and other conditions are the same 1 and 2

TABLE 2 *The Number of Inhibitors and the Inhibitory Pattern of Various Colostrum*

Proteolytic enzymes (Origin)	Colostrum																
	Human			Pig							Cattle						
Trypsin (bovine swine)	(1)*	I†		(4)	I	II		IV		VI	(2)	I	II				
α Chymotrypsin	(2)	I	II	(2)	I					VI	(1)		II				
Bacillus subtilis	(2)	I	II	(2)			III			VI	(3)	I				V	VI
Aspergillus oryzae	(0)			(1)			III				(2)	I					VI
Bacillus cereus	(0)			(1)		II					(?)			III			VI
Corynebacterium pyogenes	(0)			(1)					V		(2)			III	IV		

* Number of inhibitors observed for each enzyme

† The different inhibitor fractions active upon each enzyme

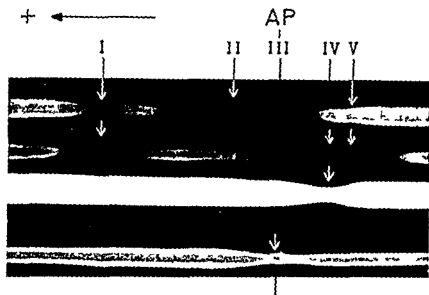


Fig 5 Electrophoretic CPI test of dog colostrum The enzymes used are (downwards) Swine trypsin α-chymotrypsin proteinase from *Bacillus cereus* and ficin The strength of the enzymes used and other conditions are the same as in Figs 1 and 2

these enzymes are the ones most affected α Chymotrypsin is also strongly inhibited, and dog colostrum inhibits this enzyme to a greater extent than it inhibits trypsin Most of the microbial enzymes tested are inhibited to some extent by colostrum from animal species The proteinase from *Corynebacterium pyogenes* is inhibited very strongly by pig colostrum and also by colostrum from cattle and goat The plant proteinases ficin and papain show a similar degree of inhibition and they are inhibited to a small degree by colostrum from all the species tested

In Figs 4 and 5 the electrophoretic patterns of the inhibitors from colostrum of cattle and dog upon some proteolytic enzymes are shown Table 2 presents a summary of the number of inhibitors found in each of the colostrum species tested against different enzymes and an indication of the specific inhibitor fraction involved It is evident that the colostrum from each species contains a complex of inhibitory substances with different inhibitory spectra For ficin and papain there was usually a zone of inhibition near the line of application This inhibitor had in some cases a migration rate similar to one of the others In other

:ies upon Different Proteolytic Enzymes Based upon the Electrophoretic CPI Test

ies

Sheep	Goat	Hor e	Dog
2) I III	(2) I II	(3) I II IV	(3) I II V
1) I	(1) I	(3) I II IV	(?) I IV or V
1) I	(3) I IV V	(4) I III IV VI	(0)
2) I II	(3) I IV V	(2) I VI	(0)
1) II	(0)	(2) III V	(1) IV
	(?) III IV	(1) III	(0)

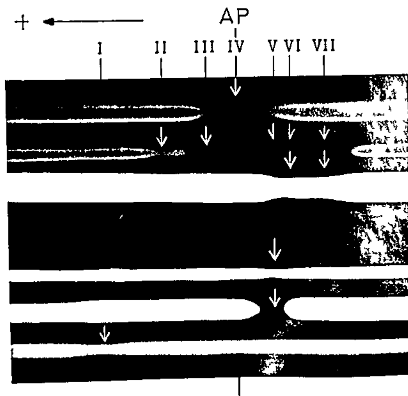


Fig 6 Electrophoretic CPI test of extract from *Ascaris suum* The enzymes used are (downwards) Swine trypsin α-chymotrypsin proteinases from *Bacillus subtilis Aspergillus oryzae Bacillus ce eus* and *Staphylococcus aureus* The strength of the enzymes used and other conditions are the same as in Figs 1 and 2 except that the electrophoresis was run for 24 hours

cases for example with dog milk the inhibitor seems to be specific for ficin (Fig 5) and papain Bovine and swine trypsins were found to be equally affected by all the different inhibitors

Colostrum from different individuals of the same species did not always show the same inhibitory pattern For example the colostrum from a second cow not shown in Fig 4 had two anodic inhibitors for the proteinases of *Bacillus subtilis* and *Aspergillus ory ae* while the most prominent inhibitor for *Corynebacterium pyogenes* (IV in Fig 4) was absent Of the two proteinase fractions

TABLE 3 *Highest Dilution of Extracts from Wheat, Barley, Rye and Corn Flours and Potato Resulting in Inhibition of Different Proteolytic Enzymes Based upon the Crosswise CPI Test*

Proteolytic enzymes (Origin)	Extracts from				
	Wheat	Barley	Rye	Corn	Potato
Trypsin (bovine and swine)	1:10*	1:10	1:200	1:10	1:500
α-Chymotrypsin	1:10	1:10	1:200	1:50	1:500
Bacillus subtilis	1:200	1:200	1:100	1:50	1:500
Aspergillus oryzae (crude)	1:200	1:200	1:100	1:50	1:200
Aspergillus oryzae (Fraction I)	1:200	1:200	1:200	1:50	1:200
Aspergillus oryzae (Fraction II)	0†	0	0	0	0
Bacillus cereus	0	0	0	1:10	0
Ficin and papain	1:10	1:10	1:10	1:10	1:10

* The extracts were diluted 1:1, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000 in saline.
† 0 = No inhibition observed.

separated from the crude *Aspergillus oryzae* proteinase only Fraction I was inhibited.

Inhibitors from Ascaris Suum

The electrophoretic patterns of the inhibitors from *Ascaris suum* upon different enzymes are shown in Fig. 6. By electrophoresis of different dilutions of the extracts used, as well as by developing with different enzyme dilutions, it was shown that α-chymotrypsin was inhibited by four electrophoretically distinguishable inhibitors (II, III, V or VI, and VII). Only one inhibitor could be demonstrated for bovine and swine trypsins. The anodic inhibitor for *Staphylococcus aureus* proteinase was found in all the extracts. None of the other enzymes tested were inhibited in this area. It was shown by the crosswise CPI test that the proteinases from *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were inhibited to approximately the same extent, while the proteinases from *Corynebacterium pyogenes*, *Proteus vulgaris*, *Serratia marcescens* and Fraction II from *Aspergillus oryzae*, as well as ficin and papain, were not inhibited.

Inhibitors in Plant Materials

Table 3 presents the inhibitory activity of extracts of wheat, barley, rye and corn flour and of potato upon different enzymes. The other bacterial enzymes included in these investigations (proteinases from *Corynebacterium pyogenes*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Serratia marcescens*) were not inhibited. It can be seen that the inhibitory substances in wheat and barley flour are much more active against the proteinases from *Bacillus subtilis* and *Aspergillus oryzae* than against the animal proteinases. In addition, of the two proteinase fractions from the crude enzyme preparation of *Aspergillus oryzae*, one is strongly inhibited by all these extracts while the other is not affected. The *Bacillus cereus* proteinase is affected only by corn extract.

The electrophoretic patterns of the inhibitors in wheat flour extract are shown in Fig. 7. Several inhibitors with different migration rates and different inhibitory spectra seem to be present, although the electrophoretic separation is not very distinct. While in rye flour two cathodic migrating inhibitors could be seen for swine trypsin, one additional anodic migrating inhibitor could be found for bovine trypsin. Altogether seven inhibitors could be demonstrated in rye flour. Barley and corn flours were also shown to contain two or more electrophoretically distinguishable inhibitors.

The electrophoretic spectra of inhibitors in potato are shown in Fig. 8. It should be

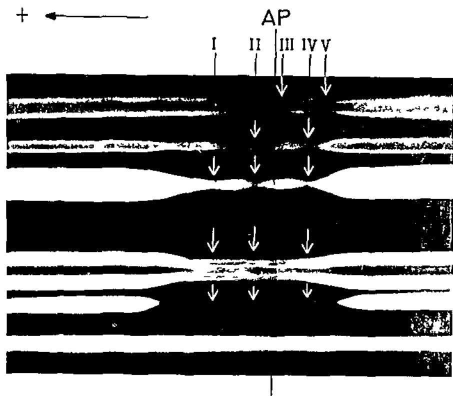


Fig 7 Electrophoretic CPI test of extract from wheat flour The enzymes used are (downwards) Swine trypsin α-chymotrypsin proteinases from *Bacillus subtilis* and *Aspergillus oryzae* (concentrations of the enzymes as in Fig 1) and Fractions I and II from *Aspergillus oryzae* The other conditions are the same as in Fig 2 except that the electrophoresis was run for 24 hours

emphasized that in this case the strength of the enzymes used for developing is 10 × greater than that used in the other figures This enzyme concentration gave a better differentiation of the zones of inhibition The presence of four different inhibitors was indicated by electrophoresis of different dilutions of the extract and by developing with different enzyme dilutions The spectra of inhibitors include one for swine trypsin α chymotrypsin and *Bacillus subtilis* proteinase (I) which does not seem to inhibit bovine trypsin one for *Bacillus subtilis* proteinase (II) one which inhibits trypsin and α chymotrypsin (III) and one which inhibits all these enzymes (IV) While bovine trypsin is inhibited to approximately the same extent by the two cathodic inhibitors (III and IV) swine trypsin is much more affected by the form r

DISCUSSION

The number of different inhibitors in the various materials tested in the present work has been determined on the basis of the migration properties of the inhibitory substances in one single electrophoretic system only Thus when zones of inhibition for several enzymes occurred at the same distance from the line of application the inhibition has been considered to be a result of one single inhibitor This may be so in many cases but it is probable that such zones can result from more than one inhibitory substance even when only one enzyme is affected (*Fossum* in press) In spite of this the present work clearly supports and extends the ideas about the complexity of the naturally occurring proteinase inhibitors

In chicken egg white three different inhibitors have previously been described the

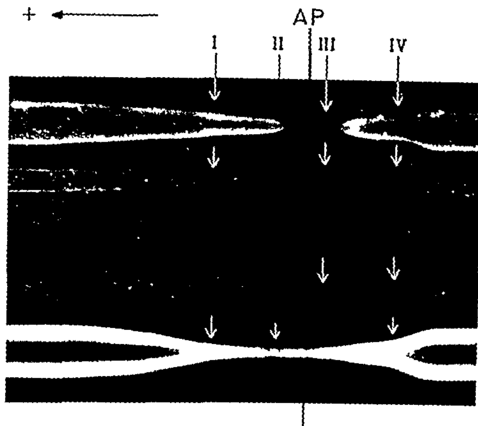


Fig 8 Electrophoretic CPI test of extract from potato The enzymes used are (downwards) Swine trypsin (0 05 mg per ml) α-chymotrypsin (0 05 mg per ml) bovine trypsin (0 1 mg per ml) and proteinase from *Bacillus subtilis* (0 01 mg per ml) The other conditions are the same as in Fig 2

ovomucoid trypsin inhibitor which inhibits trypsin only (*Feeney et al* 1963) ovoinhibitor which inhibits trypsin chymotrypsin and some microbial enzymes (*Matsushima* 1958 *Feeney et al* 1963) and a ficin and papain inhibitor which also inhibits bromelain to some extent but does not inhibit trypsin α-chymotrypsin nor a variety of microbial enzymes (*Fossum* & *Whitaker* 1968)

The present work shows that most of the proteolytic enzymes investigated are inhibited to some extent by substances in egg white and the electrophoretic CPI test indicates the presence of several inhibitors with different inhibitory spectra Based on similarities with the ovomucoid trypsin inhibitor as far as electrophoretic migration and inhibitory spectrum are concerned the anodic trypsin inhibitor (II in Fig 2) is thought to be caused, at least partly by the ovomucoid trypsin inhibitor The faster moving inhibitory substance, which seems to be active only against swine trypsin and not against bovine trypsin (I) indicates that the two trypsins are not equally affected by all the inhibitors The zones of inhibition for trypsin α chymotrypsin proteinase from *Bacillus subtilis* and Fraction I from *Aspergillus oryzae* (V) are thought to be caused by the ovoinhibitor based on the inhibitory spectrum (*Matsushima* 1958 *Feeney et al* 1963) The residual proteolytic activity of *Aspergillus oryzae* proteinase in this area seems to be due to the activity of Fraction II because this enzyme fraction was found to be unaffected while Fraction I was inhibited It is of interest that the bacterial proteinases except that of *Bacillus subtilis* were not inhibited in this area

The anodic zones of inhibition affecting

all the enzymes tested except trypsin seem to be caused by inhibitory substance(s) (III) not previously described although this seems to represent the only inhibitory substance(s) in chicken egg white against many bacterial proteinases The electrophoretic migration indicates the presence of one polyvalent inhibitor, active upon several animal, microbial and plant proteinases The existence of several inhibitory substances with similar migration rates cannot be ruled out until purification of the factor(s) has been performed The slower moving anodic inhibitor for ficin which also inhibits papain is considered to be the ficin and papain inhibitor reported by *Fossum* & *Whitaker* (1968) based on electrophoretic migration (*Fossum* 1967) and inhibitory pattern of the substance

The weak zone of inhibition for trypsin and *Bacillus subtilis* proteinase near the line of application as shown by the electrophoretic CPI test of ovomucoid trypsin inhibitor (Fig 3) may be due to contamination with ovoinhibitor This is in accordance with the findings of *Feeney et al* (1963) The fact that contaminating substances with inhibitory activity can be demonstrated by the electrophoretic CPI test in highly purified material supports the previously reported high sensitivity of the CP and CPI tests (*Sandvik* 1962 *Fossum* 1970)

The colostrum inhibitors are of interest because of their supposed physiological importance as protective agents for the immune bodies in the colostrum against tryptic degradation in the intestinal tract of the newborn animals (reviewed by *Vogel et al* 1968) The nature of the placentation in some animals for example cattle goat sheep pig and horse may prevent the transfer of maternal immune bodies into the fetal circulation while for example in human a prenatal transmission of passive immunity takes place (*Humphrey* & *White* 1966) It is of interest that the human colostrum shows less inhibitory activity upon trypsin (bovine and swine) than does the colostrum from the animal species tested (Table I) The present results are in accordance with an earlier observation which indicated that the relative trypsin inhibitory capacity in human cow, and sow colostrum are present in the proportion 1 10 67 (*Laskowski et al* 1957)

Although a trypsin inhibitor has been crystallized from bovine and swine colostrum (*Laskowski* & *Laskowski* 1951 *Laskowski et al* 1957) little seems to be known about the complexity of these inhibitors as far as inhibitory spectrum and numb r of existing inhibitors are concerned The crystalline trypsin inhibitor from bovine colostrum was found to inhibit α chymotrypsin nonstoichiometrically (*Wu* & *Laskowski* 1955) but no data have been found with respect to the effect upon microbial enzymes The present work indicates that a great variety of inhibitors is present in colostrum from different animal species

The possible relationship between serum inhibitors, and inhibitors in colostrum has not been investigated The di nce in electrophoretic migration as well a in inhibitory spectrum (*Fossum* in press) seem however to indicate different inhibitory substances in the two systems The high inhibitory activity against *Corynebacterium pyogenes* proteinase in colostrum from some individuals of cattle pig and goat as well as the electrophoretic migration of the main inhibitor for this proteinase indicates the presence of specific antibodies in the colostrum due to a previous bacterial infection

Green (1957) showed indirectly by inactivation studies that the inhibition of trypsin and chymotrypsin in extracts of body wall of *Ascaris suum* was due to different inhibitors *Rhodes et al* (1963) found by means of carboxymethyl cellulose chromatography one trypsin inhibitor and two chymotrypsin inhibitors in the body wall and perienteric fluid *Rola* & *Pudles* (1966) demonstrated by means of cellulose acetate electrophoresis three components having inhibitory activity against chymotrypsin The specific trypsin inhibitor has also been purified (*Pudles et al* 1967) In the present work four inhibitors of α-chymotrypsin could be demonstrated It was also evident from

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Acta path microbiol scand Section B 78 755–759 1970

PROTEOLYTIC ENZYMES AND BIOLOGICAL INHIBITORS

IV *Bacterial Proteinase Inhibitors and their Effect upon Enzymes of Various Origin*

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Extracts from disintegrated cells of some Gram negative bacteria including *Proteus mirabilis* *Proteus vulgaris* *Escherichia coli* a *Klebsiella* species *Serratia marcescens* and *Pseudomonas aeruginosa* were shown to inhibit the activity of trypsin α chymotrypsin and the extracellular proteinases of certain microorganisms as tested by the casein precipitating inhibition test (CPI test) The crude extracts of the various proteolytic Gram negative bacteria did in all cases inhibit the extracellular proteinase produced by the homologous species Ficin and papain were not inhibited by any of the bacterial inhibitors tested Disintegrated cells of Gram positive microorganisms showed no inhibitory activity By electrophoresis of the inhibitor containing material combined with the CPI test (electrophoretic CPI test) two electrophoretically distinguishable inhibitors were demonstrated from the *Proteus* bacteria one which inhibited only trypsin and α chymotrypsin and another which in addition to affecting these two enzymes to some extent also inhibited microbial proteinases Only one inhibitor was demonstrated by this method from each of the other Gram negative bacteria The inhibitors which are assumed to be proteins were non dialysable they were precipitable by ammonium sulphate but not by streptomycin sulphate They were inactivated by the action of pepsin and were fairly heat stable in the crude preparation

Naturally occurring inhibitors against proteolytic enzymes are present in several materials of animal and plant origin The complexity of the inhibitors as far as the number of inhibitory substances and the inhibitory pattern is concerned has been investigated by a combination of paper electrophoresis of different inhibitor containing substances and the casein precipitating inhibition test (electrophoretic CPI test) (*Fossum* In press a b)

There are few reports concerning trypsin inhibitors of bacterial origin *Hojem & Skulberg* (1962) reported the presence of a trypsin inhibiting factor in culture supernatants of *Clostridium botulinum* Recently *Brecher & Pugatch* (1969) reported a non-dialysable heat stable trypsin and chymotrypsin inhibitor in extracts of sonicated cells of *Escherichia coli*

The aim of the present work was to examine different species of bacteria for the

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PROTEOLYTIC ENZYMES AND BIOLOGICAL INHIBITORS

IV *Bacterial Proteinase Inhibitors and their Effect upon Enzymes of Various Origin*

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Extracts from disintegrated cells of some Gram negative bacteria including *Proteus mirabilis* *Proteus vulgaris* *Escherichia coli* a *Klebsiella* species *Serratia marcescens* and *Pseudomonas aeruginosa* were shown to inhibit the activity of trypsin α chymotrypsin and the extracellular proteinases of certain microorganisms as tested by the casein precipitating inhibition test (CPI test) The crude extracts of the various proteolytic Gram negative bacteria did in all cases inhibit the extracellular proteinase produced by the homologous species Ficin and papain were not inhibited by any of the bacterial inhibitors tested Disintegrated cells of Gram positive microorganisms showed no inhibitory activity By electrophoresis of the inhibitor containing material combined with the CPI test (electrophoretic CPI test) two electrophoretically distinguishable inhibitors were demonstrated from the *Proteus* bacteria one which inhibited only trypsin and α chymotrypsin and another which in addition to affecting these two enzymes to some extent also inhibited microbial proteinases Only one inhibitor was demonstrated by this method from each of the other Gram negative bacteria The inhibitors which are assumed to be proteins were non dialysable they were precipitable by ammonium sulphate but not by streptomycin sulphate They were inactivated by the action of pepsin and were fairly heat stable in the crude preparation

Naturally occurring inhibitors against proteolytic enzymes are present in several materials of animal and plant origin The complexity of the inhibitors as far as the number of inhibitory substances and the inhibitory pattern is concerned has been investigated by a combination of paper electrophoresis of different inhibitor containing substances and the casein precipitating inhibition test (electrophoretic CPI test) (*Fossum* In press a, b)

There are few reports concerning trypsin inhibitors of bacterial origin *Høyem* & *Skulberg* (1962) reported the presence of a trypsin inhibiting factor in culture supernatants of *Clostridium botulinum* Recently *Brecher* & *Pugatch* (1969) reported a non dialysable heat stable trypsin and chymotrypsin inhibitor in extracts of sonicated cells of *Escherichia coli*

The aim of the present work was to examine different species of bacteria for the

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possible presence of intracellular inhibitors and, by use of the electrophoretic CPI test, to study the complexity of these bacterial inhibitors.

MATERIALS AND METHODS

The following enzymes were obtained from Sigma[1]: Trypsin from bovine pancreas (Type III, 2 x crystallized, lot 97B-8000), α-chymotrypsin from bovine pancreas (Type II, 3 x crystallized, lot 86B-0470), protease from *Bacillus subtilis* (Type VIII, crystallized, lot 17B-2770), protease from *Aspergillus oryzae* (Type II, crude, lot 13?0), ficin from fig tree latex (2 x crystallized, lot 119B-4750), papain from papaya latex (? x crystallized, lot 4?B-10?0). Trypsin from swine pancreas (crystallized, batch No 36476) and pepsin from swine stomach mucosa (4489t, 3 x crystallized) were obtained from Koch Light Laboratories Ltd[2]. The other microbial enzymes were prepared as described previously (*Fossum In press* a). Streptomycin sulphate was obtained from Glaxo[3] (Streptomycin Glaxo).

Preparation of Bacterial Inhibitors. Most strains of the microorganisms used have been isolated and classified by this department. The organisms investigated for inhibitory activity were a) usually cultured for two days at 30–37 C on nutrient agar in Petri dishes, after which the cells were washed off, or b) inoculated in 30 ml of saline onto the surface of 50 ml of a 2 per cent nutrient agar in 500 ml Erlenmeyer flasks, after which the flasks were incubated in a rotary shaker, usually for two days at 30–37 C, and the organisms collected by centrifugation. The cells were in each case washed 3 – 4 times in saline and, if necessary, stored at -25 C before disintegration was carried out. Cell disruption was performed either by use of an X-press[4], a French pressure cell[5], or by a Branson nifier cell disruptor[6].

The X-press usually gave the highest degree of cell disruption, as tested by microscopic examination, especially for Gram positive bacteria.

The disintegrated material was, after dilution in saline, centrifuged at 75000 g for one hour in some cases. The supernatant was either used directly, or the inhibitor-containing material was precipitated by the addition of ammonium sulphate to 80 per cent saturation at +4 C, after which the precipitate was collected and dialysed against distilled water. The supernatant was sometimes heated to temperatures between 70 and 100 C, either prior to or after precipitation with ammonium sulphate and dialysis.

Washed cell suspensions were also boiled for 2–5 minutes in an attempt to extract the inhibitors from the cells.

The digestibility of the inhibitors by pepsin was tested by adding the enzyme (0.01 mg) to 1 ml of the crude material after adjustment of the pH to 2.0. After incubation at 37 C for 30 minutes and neutralization, evaluation of the inhibitory activity was carried out. Ficin digestibility was tested at pH 7.0 by adding the enzyme (0.01 mg to 1 ml of extract) together with sulfhydryl activators, and after incubation at 37 C for 30 minutes the inhibitory activity was determined.

Streptomycin sulphate was added to aliquots of crude material as a 10 per cent solution (w/v) under constant stirring over a period of 10 minutes to give a final concentration of 15 mg per ml. The inhibitory activity in the supernatant was then evaluated.

Determination of Inhibitory Activity. The crosswise CPI test was performed as described by *Fossum* (1970, *In press* a), using as substrate sodium caseinate incorporated into agar. Electrophoretic differentiation of the inhibitors in the various extracts was carried out in a way similar to that for sera and other inhibitor-containing materials (*Fossum In press* a, b). Usually two or more strains of each species were examined for inhibitory activity.

RESULTS

The inhibitory effects of some bacterial extracts upon various proteolytic enzymes, based on the crosswise CPI test, are shown in Table 1. It is seen that trypsin, α-chymotrypsin and proteinases from *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis*, *Corynebacterium pyogenes* and *Aspergillus oryzae* are inhibited by all the bacterial extracts listed in the table, while proteinases from *Proteus* bacteria are inhibited only by *Proteus* extracts and the proteinase from *Serratia marcescens* only by extracts from *Serratia* and *Proteus* cells. Extracts of *Proteus vulgaris* and *Proteus mirabilis* behaved in a similar manner in all respects. Ficin and papain were not inhibited by any of the bacterial extracts.

Boiling of cell suspensions of Gram negative bacteria resulted in the liberation of inhibitory factors.

[1] Sigma Chemical Company, St. Louis, Mo, U.S.A.

[2] Koch Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

[3] Glaxo Laboratories Ltd., Greenford, England.

[4] AB Biox, Nacka, Sweden.

[5] American Instrument Company Inc., Silver Spring, Md, U.S.A.

[6] Branson Sonic Power, 51 Mry Brook Road, Danbury, Conn., U.S.A.

TABLE 1 *The Effect of Some of the Crude Bacterial Inhibitors upon Proteolytic Enzymes of Various Origin Tested by the Crosswise CPI Test*

Proteolytic enzymes (origin)	Inhibitors (origin) *Proteus vulgaris* (NVH 2674)*	*Proteus mirabilis* (NVH 2763)	*Escherichia coli* (NVH 2666)	*Klebsiella* species (NVH 2764)	*Serratia marcescens* (NVH 2672)	*Pseudomonas aeruginosa* (NVH 982)
Trypsin (bovine and swine)	++++ †	++++	++++	++++	++++	++++
Chymotrypsin	++++	++++	+++	++++	++++	++++
Proteus vulgaris (NVH 2674)	++++	++++	– §	–	–	–
Proteus mirabilis (NVH 2763)	++++	++++	–	–	–	–
Serratia marcescens (NVH 2672)	++++	++++	–	–	++++	–
Pseudomonas aeruginosa (NVH 982)	++++	++++	++++	++++	++++	++++
Bacillus cereus (NVH 322)	++++	++++	+++	++	++	++++
Bacillus subtilis	++++	++++	+++	++	++	++++
Corynebacterium pyogenes (NVH 431)	+++	+++	+++	++	++	++++
Aspergillus oryzae	+++	+++	++	++	++	++
Ficin and papain	–	–	–	–	–	–

* NVH The Culture Collection of the Department of Microbiology and Immunology Veterinary College of Norway

† ++++ +++ ++ Various degree of inhibition § No inhibition observed

Extracts of disintegrated or boiled cells of Gram positive bacteria including *Staphylococcus aureus* *Staphylococcus epidermidis* a *Micrococcus* species and *Bacillus cereus* did not inhibit any of the enzymes tested including the proteinases of the homologous species Disintegration of yeast cells resulted in activation or liberation of proteolytic enzymes No inhibitors could be demonstrated in these materials

Culture supernatants and whole cell suspensions of the various microorganisms did not cause any inhibition of the proteolytic enzymes tested After freezing and thawing of washed cell suspensions of Gram negative bacteria inhibitory activity was demonstrated in some cases

The electrophoretic patterns of the inhibitors in extracts of *Proteus mirabilis* are shown in Fig 1 Two electrophoretically distinguishable inhibitors can be seen one which inhibits trypsin and α chymotrypsin (I) and another which in addition to inhibiting these two enzymes to some extent also affects the microbial enzymes (II) Extracts from *Proteus vulgaris* showed a similar picture The electrophoretic CPI test performed with the extracts of other Gram negative bacteria revealed only one inhibitor

Bovine and swine trypsins were equally affected by the bacterial inhibitors as far as could be seen by the method used The bacterial inhibitors were acid stable (pH 2 0 for 1 hour at 37°C) they lost their activity by the action of pepsin but not by ficin On addition of streptomycin sulphate and subsequent centrifugation at 20000 g the inhibitory activity was found to be present in the supernatant The inhibitors were precipitated at 80 per cent saturation with ammonium sulphate and were non dialysable Heating of the crude material to 70–100°C for 2–5 minutes did not result in any major inactivation of the inhibitors

DISCUSSION

The naturally occurring proteinase inhibitors which have been purified have proved to be

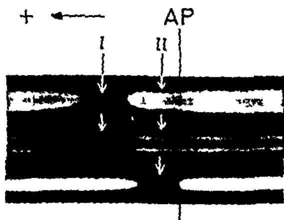


Fig 1 Electrophoretic CPI test of extract from *Proteus mirabilis* The enzymes are (downwards) Swine trypsin and α chymotrypsin (0 005 mg per ml) and proteinase from *Proteus mirabilis* Electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 24 hours The electrophoretically distinguishable inhibitors resulting in zones of inhibition are numbered from left to right and the zones of inhibition are marked by arrows

either proteins or proteins combined with a polysaccharide (reviewed by *Vogel et al* 1968) or with sialic acid (*Astrup & Nissen* 1964) Bacterial inhibitors have not yet been purified and the nature of these inhibitors is therefore unknown The inhibitors described in the present paper differ from most of the previously described inhibitors of animal and plant origin by being strictly intracellular substances. Bacterial cells differ from animal and plant cells in their high content of ribonucleic acids the presence of D amino acids in the cells and in the structure of the cell wall among other differences. Whether these peculiarities have any influence upon the inhibitory activity has not yet been elucidated The precipitation and peptic digestibility experiments carried out indicate that the bacterial proteinase inhibitors are also proteins although their heat resistance is higher than that for most other proteins. In this connection it is noteworthy that some of the naturally occurring proteinase inhibitors are remarkable resistant to heat (*Lineweaver & Murray 1947 Laskowski & Wu 1953 Green* 1957 *Kiermeyer & Semper* 1959/60 *Astrup & Nissen* 1964 *Ryan* 1966 *Fossum & Whitaker* 1968) The inability to precipitate the inhibitors with streptomycin sulphate seems to exclude ribosomal nucleic acids as being of importance for the inhibitory activity (*White et al* 1968)

The inhibitors described are in many respects, similar to the one isolated from *Escherichia coli* cells reported by *Brecher & Pugatch* (1969) although their studies only included the inhibitory activity against trypsin and chymotrypsin

The observation that some of the inhibitors from extracellular proteinase producing bacteria inhibit the cell's own proteinase is interesting and could lead to the possibility that a function of the inhibitors is as a regulating or protecting factor This hypothesis may be supported by the supposed function of the inhibitors of *Ascaris suum* as a defence mechanism against the proteinases of the host organism (*Vogel et al* 1968) It should be emphasized in this connection that the natural residence of most of the bacteria investigated in the present work is the human or animal intestine

The proteolytic activity observed in extracts of disrupted yeast cells is in accordance with the knowledge that proteinases are contained in the yeast cells and can be liberated by autolysis of the cells (reviewed by *Harris* 1958) or by mechanical destruction of the cells (*Hayashi et al* 1968)

By use of the electrophoretic CPI test two distinguishable inhibitors were observed in *Proteus* extracts and only one in the extracts of the disintegrated cells of the other Gram negative bacteria tested However the existence of more than the one or two inhibitors found here cannot be eliminated on the basis of the present work

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CHEMICAL COMPOSITION OF ENDOTOXIN FROM ORAL *VEILLONELLA*

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Lipopolysaccharide (LPS) has been prepared from four human oral strains of *Veillonella* by phenol water extraction of acetone washed whole cells. LPS from all strains contained 2 keto 3-deoxy octonate (KDO), heptose, glucose, glucosamine and galactosamine. In addition, galactose was present in one strain, and galactose and ribose in another. The preparations were rich in lipid and KDO.

Serologically specific lipopolysaccharide (LPS) with a high degree of endotoxic activity has been prepared from oral strains of *Veillonella* (8, 14, 15, 21). Little is known of the chemistry of this LPS. Phenol water extracted LPS from one single strain was examined for sugar components by thin layer chromatography of acid hydrolysates (13). The components found were galactosamine, glucosamine, glucose and fucose. In an earlier study (16) the isolated LPS was found to contain a thiobarbituric acid reactive component. Lipid accounted for the major portion of LPS from this and other *Veillonella* strains (15).

The present paper deals with the chemical composition of phenol water extracted LPS from four oral strains of *Veillonella*. We were especially interested to learn whether this highly active endotoxin contained heptose and 2 keto 3 deoxy octonate (KDO) or not. Atypical LPS lacking these sugars have recently been isolated from *Neisseria catarrhalis* (2) and from *Bacteroides* (6, 7).

MATERIALS AND METHODS

The *Veillonella* strains Ve5, Ve6, Ve8 and Ve9 were isolated from saliva of four human adults. The strains fermented lactate with production of gas and grew anaerobically on Bacto Veillonella agar with typical smooth colonies.

Bulk cultivation was performed in 500 ml screw cap bottles filled to the top with the following medium: Bacto-Tryptone 5 g, Bacto Yeast Extract 3 g, sodium thioglycolate 0.75 g, sodium lactate 60 per cent 21 ml, distilled water to make 1 l, pH adjusted to 7.5. Strains Ve6, Ve8 and Ve9 grew with a uniform turbidity of the medium while strain Ve5 grew more slowly as a granular deposit. After one or two (Ve5) days incubation at 37 C the bacteria were harvested by centrifugation, washed three times in saline and dried with acetone.

Isolation of LPS. Extraction of LPS with phenol ... (26) and further purification by ultracentr... on were carried out as described earlier ... actions were made at room temperature for 15 min an aqueous suspension of 50 ... e dried bacteria per ml with an equal

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volume of 90 per cent phenol The water phase was recovered after centrifugation (2 500 g for 30 min) After ultracentrifugation (100 000 g for 1 hour) the pellet was treated with ribonuclease and deoxyribonuclease (7) In some experiments the extraction was repeated with the same volume of distilled water LPS prepared from the first aqueous supernatant is termed LPS I while the material recovered from the second extraction is termed LPS-II

Paper Chromatography Acid hydrolysis was performed in sealed tubes at 100 C with 3 N HCl for 3 hours with 0 1 N H_2SO_4 for 10 min or with N H_2SO_4 for 10 min 4 or 16 hours Acid was removed from HCl hydrolysates by evaporation *in vacuo* at room temperature in the presence of NaOH pellets Salt free preparations were made from the 0 1 N H_2SO_4 hydrolysates by neutralization with $Ba(OH)_2$ followed by centrifugation and evaporation of the supernatant to dryness *in vacuo* The other H_2SO_4 hydrolysates were neutralized by passage through a column of Dowex 1 in the formate form Circular paper chromatography was carried out with *n* butanol pyridine water (6 4 3) or phenol water (4 1) Reducing sugars were stained with silver nitrate or aniline hydrogen phthalate Amino sugars were also detected with the Elson Morgan reagent (20) The Warren thiobarbituric acid reagent (24) was used to detect KDO

Chemical Analyses Nitrogen was determined by the micro Kjeldahl method as described in (9) Samples were digested for 4 hours The Folin Ciocalteau phenol method (11) was used for estimation of protein Bovine serum albumin served as standard Fatty acid esters were determined as tripalmitin by the method of *Snyder & Stephens* (23) Hexosamine was estimated by the *Rondle & Morgan* method (22) with glucosamine HCl as standard Neutral sugars were measured by the *Hm ler* orcinol method (27) with gl c e galactose (1 1) as standard The presence of h ptos was demonstrated by the sulphuric ac d [illegible] action of *Dische* (4) KDO was det [illegible] the thiobarbituric acid method a [illegible] *Weis bach & Hurwitz* (25) Sample [illegible] sed in 0 02 N H_2SO_4 for 20 min at 100 C [illegible] KDO standard prepared from a [illegible] 2 4 5 8 penta O acetyl KDO methyl e te [illegible] by *Dr Eduard C Heath* Baltim [illegible] the method of *Ghalambor et al* (5 [illegible]

RESULT

Quantitative Analyses Re ults [illegible] uantitative chemical analyses of two batc [illegible] S I a one batch of LPS II from eac [illegible] e fou strains of *Veillonella* have been c [illegible] d in Table 1 The figures represent me [illegible] alues of 3-6 determinations LPS isolated by extraction for 15 min (LPS I) of strains Ve5

TABLE 1 *Percentage Chemical Composition of LPS Isolated from 4 Strains of Human Oral* Veillonella *by Extraction of Acetone Dried Cells with Phenol Water*

		Yield*	Neutral sugar	Hexosamine	KDO	Fatty acid ester	Protein	Nitrogen
Strain Ve5								
Batch 1	LPS I	2 0	30 7	7 7	9 5	30 2	7 7	2 4
2	LPS I	2 5	27 3	5 4	8 4	38 5	2 2	2 0
	LPS II	1 7	21 0	5 8	5 8	19 9	11 7	6 6
Strain Ve6								
Batch 1	LPS I	2 0	20 5	12 2	9 4	30 4	2 3	3 5
2	LPS I	1 7	22 0	7 9	8 4	34 0	2 9	2 2
	LPS II	1 6	20 0	10 4	11 [illegible]	39 0	5 6	2 2
Strain Ve8								
Batch 1	LPS I	2 0	28 [illegible]	6 5	7 7	20 6	24 4	5 5
	LPS-I	2 0	31 1	8 0	6 0	26 [illegible]	12 6	3 8
	LPS II	1 1	39 9	[illegible] 2	9 1	29 6	5 4	2 3
Strain Ve9								
Batch 1	LPS I	2 0	15 3	8 2	9 0	33 3	3 7	2 1
2	LPS I	1 4	17 6	5 8	7 2	34 [illegible]	4 9	3 0
	LPS-II	0 7	12 4	6 4	7 5	43 3	3 0	2 4

Per cent of acetone dried cells

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ELECTRON MICROSCOPICAL AND SEROLOGICAL CHARACTERISTICS ASSOCIATED WITH COLONY TYPE IN *MORAXELLA NONLIQUEFACIENS*

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Pairs of the spreading corroding (SC) colonies on the one hand and normal m u (N) colonies on the other of *Moraxella nonliquefaciens* were studied Electron m c re vealed that SC cultures are distinguished by the occurrence of numerous fimbr (hi) of 60–70 Å diameter The usual SC fimbriae are morphologically different fro r filament type found occasionally around cells from N colonies and detected very r re in SC cultures Serologically no easily observed difference was found between SC and N of the same origin but the former was distinctly prone to incomplete agglutination in anti sera against the SC type absorbed by cells of the other type This could be ascribed to the fimbriae which were probably present in unequal quantities among the cells of SC populations. In both colony variants a heat labile envelope antigen corresponding to the *B* type was discovered The possible roles of the fimbriae are briefly discussed

The existence of corroding and spreading colony forms of *Moraxella nonliquefaciens* was recently reported by *Henriksen & Bovre* (16) In one strain examined the spreading corroding property was a stable trait as was *also the normal smooth appearance of other* variants

The present investigation was originally centered upon one strain 3067/66 which exhibited three different colonial forms Both the spreading corroding variant (here called the SC type) and the normal non spreading non corroding form (here called the N type) appeared upon the first subcultures from a single colony isolate and remained stable in subsequent serial subcultures of the SC and the N populations As an exception in SC cultures an intermediate phenotype (named the NSC type) was discovered An NSC colony will regularly throw off a low percentage of typical stable SC colonies among its *descendants The N type of this strain thus* far seems to be permanent

This system of two stable colony variants and on intermediate unstable substrain of the sam or in has induced the present attempt to sea ch with the electron microscope for morpho ogical hall marks of the colonial variants of this and other strains at the cellular level and to examine whether the differences are reflected in serological reactions Together with a physiological and biochemical definition of the type variation which is only preliminarily reported here the study is planned to form the basis for later attempts

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at exact genetic analysis of the phenotypic parameters.

MATERIALS AND METHODS

Bacterial Strains

In addition to the colony forms of strain 3067/66 the stable and morphologically similar N and SC types of the strains 3179/66 3832/66 and 6088/68 were chosen for comparison in the electron microscopical studies The N and SC types of strains 3179/66 and 3832/66 were also compared with those of 3067/66 in growth experiments in semisolid and fluid media and in transformation experiments All strains had been isolated as single colonies from primary nose cultures The three first mentioned strains were isolated during a systematic study of the oxidase positive organisms in the human nose (5) The representative colonial morphology of the 6088/68 N and SC types has been presented previously (16)

Cultural and Biochemical Tests

The methods were those previously described (4 7 9 16) Blood agar cultures were used for the study of colonial characteristics Generally 33 C was used as the incubation temperature with some comparisons made at 37 C. Expression of the spreading character seemed slightly superior at the latter temperature Care was taken to ensure a high degree of humidity during incubation.

Genetic Transformation

The procedure of quantitative streptomycin resistance transformation was employed as described (3) The technique of continuous deoxyribonucleic (DNA) exposure was sometimes also used (1) As recipient in some transformation experiments was used the highly competent strain NCTC /64 (4)

Buoyant Density Determination

The methods of DNA preparation and CsCl gradient centrifugation were those described previously (8)

Electron Microscopical Techniques

The preparations examined were from blood agar cultures grown for two days at 33 C in a humid atmosphere or from Mueller Hinton Broth (Difco) cultures incubated for two or [illegible] days at the same temperature With a spatula a few typical colonies were removed from agar surface (alternatively material was collected from surface growth or sediment in the fluid medium) and suspended in approximately 100 µl buffer (0 05 M sodium acetate 0 01 M magnesium acetate pH 5 5) on a microscope slide About 25 µl of 4 per cent (w/v) sodium silicotungstate (pH 6 5) was added for negative staining the mixture stirred and some of it transferred to a 200 mesh copper grid covered by a thin carbon film The transfer was achieved by touching the liquid surface with the grid After 30 seconds duration the excess fluid was removed by touching the edge of the grid with a filter paper Subsequently the preparation was examined in a *Siemens Elmiskop 1A* operated at 80 kV at a primary magnification of 10 000 or 20 000 times For selected cases the photographic differentiation technique of *Markham et al* (20) was used to better reproduce structures within the negative stain

Antigen Preparation

Cells were taken off blood agar plates after growth in a humid atmosphere at 33 C overnight. For immunization purposes a saline suspension of optical density (O D $_{540}$ Coleman Junior Spectrophotometer 16 mm cylindrical test tubes) = 0 8 was prepared and frozen in 1 ml aliquots at −20 C until employed for immunization For agglutination suspensions were used on the day of preparation Heat treated cells were obtained by exposure to 100 C for one hour and then washed four times in saline before use The unheated cells were not washed except for some control experiments

Immunization Procedure

Biweekly intravenous inoculations were made in rabbits starting with 0 25 ml – 0 5 ml – 1 0 ml each of the three first times and thence proceeding for 2–3 months with 1 0 ml per injection

Agglutinin Absorption

Thoroughly admixed on a Vortex mixer for three minutes with an equal volume of packed cells the sera were incubated at room temperature overnight.

Agglutination Procedure

Twofold dilutions of serum proceeding from 1/10 were made in saline Volumes of 0 25 ml of serum dilution were mixed in 1 × 8 cm test tubes with equal aliquots of the cell suspensions and incubated overnight in a 37 C water bath

RESULTS

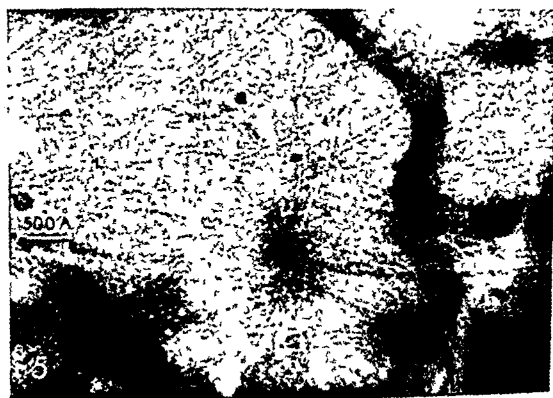
Species Diagnosis

The behaviour of the strains 3067/66 3179/66 3832/66 and 6088/68 in cultural and biochemical tests corresponded to the general characteristics of *M nonliquefaciens* (4 6) The three former strains were also proved to belong to this species by means of genetic transformation (5) at a time when the different colony forms had not yet been recognized as separable entities The buoyant density of 3067/66 DNA was found to be within the range for *M nonliquefaciens* (see below)

Emergence of the Colony Variants and Their Characteristics on Blood Agar

The strain 3067/66 was isolated from a nose specimen in which only a few oxidase positive colonies were found and no other organism A single colony was subcultured and lyophilized In the first culture from the ampoule it was noticed that approximately 80 per cent of the colonies were of the spreading type whereas the remaining ones had the ordinary, non spreading appearance A colony of the stable non spreading non corroding form (N type) and a colony of the stable spreading type which also produced a pronounced pitting of the agar beneath the colony (SC type) were subsequently isolated No spontaneous conversion has been observed from the N type of 3067/66 into the SC type in successive subcultures on blood agar for more than a year including hundreds of plates examined during the serological investigation and a special examination of more than 100 000 well separated colonies Numerous attempts to isolate spontaneous N variants from SC cultures regularly failed during serial blood agar passages. In the order of 25 000 carefully separated colonies and several hundred plates with semiconfluent growth were studied without detection of SC $\rightarrow$ N variation The N and SC variants of the strains 3179/66 and 3832/66 were easily picked in early subcultures of single colony isolates and appeared stable In the order of 30 000 separated colonies of each N form of these two strains were examined without detection of spreading However by scraping off the bacterial mass from plates with confluent growth, a few very small pits could be observed in the agar which were not seen in the 3067/66 N cultures This shows that the N forms examined of these two strains are probably not permanent contrary to 3067/66 N With a frequency of about 10^{-4} or less an intermediate colony form was discovered in late sub[illegible]tures of all three SC substrains This varia[illegible] displayed less pitting of the agar and did not spread (NSC type) In subsequent su[illegible] culture it was shown that the NSC isolate were not stable but rendered differen[illegible] proportions of descendant colonies which partly contained phenotypically stable SC cells The first NSC isolate from 3067/66 SC which is the one studied in CsCl gradients [illegible] electron microscopically and serologi[illegible] [illegible]sterly revealed the highest frequency [illegible] vari[illegible]tion observed (0 1 – 2 per cent [illegible] descendant colonies showing spreading [illegible]hots) One did not succeed in stabilizing the N type from this variant although the most N like colony was picked at each subculture By increasing the incubation time beyond the usual 2–4 days a small additional number of these NSC colonies revealed offshots of spreading growth and the agar pitting of all colonies increased somewhat Other occasional variants originating from 3067/66 SC cultures were generally not corroding but showed a very low frequency of descendant colonies with spreading offshots (0 003 per cent or less) *i e* with a very low potential for reversal to SC It is obvious that the distinction between some of these forms and the N isolates of 3179/66 and 3832/66 examined would be arbitrary It is relevant to note from work with clinical isolates of *M nonliquefaciens* (*Bøvre* unpublished) that strains which consisted of a large proportion of spreading colonies in early subcultures gradually changed to predominantly non spreading colonial forms during months of non selective subcultures performed approximately every 14th day

4



Most cells of the SC colony variant possessed numerous long filamentous extracellular appendages As shown in Fig 1 in which a positive staining effect has unintentionally been obtained the filaments clearly originate from the bacterial cells with no predominance in any particular surface area The halo with parallel filaments perpendicular to the cell body is possibly the result of shrinkage of the latter during preparation, but could also be explained by the presence of an extracellular substance The filaments are of uniform thickness and appear straight or slightly curved and of varying length When present in large numbers they were often positioned side by side in a parallel manner frequently for relatively long intervals They also occasionally formed thick bundles Fig 1 indicates a mean diameter of about 100 Å In negative stain preparations the white lines are 40–50 Å wide (Fig 3) When measuring from center to center in filaments running in parallel one finds 60–70 Å as a minimum distance and this is believed to be close to the true diameter Often the filaments were found with no cells associated Fine structure has not been detected within the filaments In a preparation from a 3067/66 SC pellicle the cells were virtually embedded in large masses of filaments (Figs 4–5)

In preparations from 3067/66 N cultures the electron microscopical picture was quite different The N type cells were never associated with large numbers of filaments In about 100 micrographs of this variant only a single filament similar to the SC filaments has been detected Occasionally however one observed filamentous structures of the type associated with the cell in Figs 6–7 In these cases the individual filaments seemed to be of a slightly larger diameter than the SC filaments but they were not so straight and always appeared fragmented seemingly fragile and it was difficult to measure them accurately Since a stable surface pellicle was not formed in broth cultures of the N variant sediment from such a culture was examined but no filament discovered

We also studied the 3067/66 NSC type from blood agar cultures, and found that it possessed filaments of the SC type in small numbers in some preparations (Fig 8) The electron microscopic pictures of the N and SC colonies of the strains 3179/66 3832/66 and 6088/68 were corroborating as regards the morphological difference between the types since the SC type filaments were never observed in N cultures, but in large numbers in most SC preparations, as shown in Figs 9–11 Once, fragmented filaments of the N type were observed among the SC type filaments in such a preparation (Fig 12)

In the SC cultures studied, we also observed some cells devoid of filamentous extracellular structures In many instances this could conceivably be explained by mechanical removal during preparation On the other hand cells were often found embedded in negative stain, displaying structures close to the cell surface of the type illustrated in Fig 13 If these cells did have filaments in a significant number we feel they would have been detected in the negative stain Such cells were observed in all three varieties

Capsular Staining

The 3067/66 N and SC variants were examined by Moller's (21) capsular staining method whereby pericellular halos developed

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Fig 4 Strain 3067/66 SC type cells from surface pellicle in Mueller Hinton Broth (Difco) (40 000 ×)

Fig 5 Strain 3067/66 SC Detail of pellicle preparation similar to Fig 4 (200 000 ×)

Fig 6 Strain 3067/66 N type filaments (40 000 ×)

Fig 7 Detail from Fig 6 (200 000 ×)

Fig 8 Strain 3067/66 NSC type cell with few fimbriae (40 000 ×)

Fig 9 Strain 6088/68 SC type fimbriae (40 000 ×)

Fig 10 Strain 3832/66 SC type cells with numerous fimbriae Photographic differentiation used (40 000 ×)

Fig 11 Strain 3179/66 SC type cell with numerous fimbriae (40 000 ×)

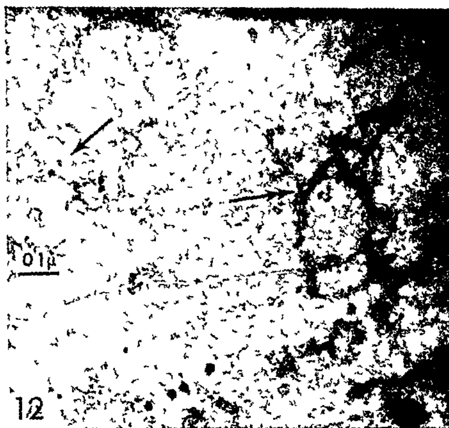

0.1μ
12



TABLE 1 *Agglutination of Strain 3067/66 of the Stable Spr ading Corroding SC) Stable Normal (N) and Unstable Intermediate Variety (NSC) with Sera against Nati e Cells* *

Antigens †	Immune sera: Anti SC$_0$ R1 § Unabsorbed	R1 Absorbed with N$_0$	R1 Absorbed with N$_t$	R2 Unabsorbed	R2 Absorbed with N$_0$	R2 Absorbed with N$_t$	Anti N: R3 Unabsorbed	R3 Absorbed with SC	R3 Absorbed with SC	R4	R4 Absorbed with SC
SC$_0$	640	0 (80–640)	0 (20–160)	320	0 (80)	0 (320)	40	0	0	[illegible]	
SC$_t$	640	0	0	320	0	0	320	0	0		[illegible]
N$_0$	80	0	0	40	0	0	40	0	0		[illegible]
N$_t$	320	0	0	320	0	0	640	0	0		
NSC$_0$	160						80				

* Titrations were performed by twofold serum dilutions starting with 1/10 10 Numbers in parentheses indicate the titer range of fluffy agglutinates with SC bid supernatant as described in the text (see Results) which appeared in fully a which the agglutinins against the absorbing cells were exhausted

† N$_0$ and N$_t$ are untreated and heat treated cells of normal non corroding colo untreated and heat treated cells of spreading corroding colonies respectively NSC the intermediate variety

§ R1–R4 denote different rabbits

TABLE 2 *Agglutinations with Crude Sera against Heat Treated Stable Spreading Corroding (SC) and Stable Normal (N) Cells of Strain 3067/66* *

Antigens †	Immune sera: Anti SC$_t$ R5 §	Anti SC$_t$ R6	Anti N$_t$ R7	Anti N$_t$ R8
SC$_0$	0	0	0	0
SC$_t$	160	320	320	320
N$_0$	0	0	0	0
N$_t$	160	320	320	320

* † The legend is identical to that of Table 1 Heterologous absorptions i e N$_t$ serum with SC$_t$ cells or SC$_t$ serum with N cells always exhausted the agglutinins against both N$_t$ and SC$_t$ cells

§ R5–R8 denote different rabbits

Fig 12 Strain 3832/66 SC type fimbriae (arrow) and N type filaments (double arrow) in the same preparation from SC colonies (100 000 ×)

Fig 13 Strain 6088/68 SC colony cell without fimbriae Photographic differentiation employed (40 000 ×)

which had the same width a o t typical nonmucoid *Escherichia co*

Serology

The results of the agglutinations in unabsorbed and absorbed sera are shown in Tables 1 and 2 The principal outcome of this part of the study is that the two extreme colony variants (N and SC) of 3067/66 are serologically very similar and that there are two distinct categories of common antigens One is thermostable and (a) initiates antibody production after injection of heat treated and possibly native cells (b) is re ponsible for agglutination of heated cells (c) is blocked for agglutination in non heated cells and (d) does absorb antibody regardless of whether the cells are heated The other component is thermolabile and consequently does not initiate homologous antibody production when immunization is done with heated cells However the antigen after heating retains the ability to absorb homologous antibody

Agglutinations were done with N and SC

cells after differentiated heat treatment in sera from all eight rabbits against either native or heat treated cells This demonstrated that heating to 60°C for one hour did not abolish the thermolabile layer Only 30 minutes at 80°C or 15 minutes at 100°C were sufficient for exposing the heat stable O antigens Cells boiled for 15 minutes usually exhibited the same titers as antigens treated for one hour whereas cells subjected to 80°C for one hour generally had slightly lower titers It shall be added that although no spontaneous clumping developed in saline suspensions a troublesome tendency towards self agglutination of the heated cells appeared in all normal sera tested This somewhat complicated the corresponding interpretations even though the morphological distinction between specific agglutination and nonspecific clumping was clear

The native N cells (N) and all the heated cells exhibited granular dense agglutinates similar to typical O agglutinates in *Pseudomonas* and enterobacteria In contrast agglutinates of the untreated SC cells (SC) were typically loose and fluffy indicating the participation of the fimbriae (see Discussion) Table 1 shows that SC cells in heterologously absorbed SC antisera frequently produced agglutinates of this particular appearance as if possessing a specific thermolabile antigen In the beginning of the study the agglutinates appeared regularly In none of these agglutinins was a clear supernatant produced however contrary to the findings with unabsorbed where agglutinations always resulted in clear supernatants Subsequently in some of the 23 absorbed sera employed the specific SC reactions were occasionally missed It was also on two occasions observed that incompletely SC absorbed N_0-antisera reacted with SC_0 cells subsequent to complete exhaustion of N agglutinins SC_0 cells as well as N cells were always stable in non immune sera

DISCUSSION

Since the N and SC variants of the strain 3067/66 appeared among descendants of a single colony from a primary nose culture with few organisms, there is an *a priori* indication of the identical cellular origin of the two colonial types Coexistence of two different strains however could not be excluded although the emergence after numerous subcultures of the morphologically intermediate strains from SC cultures links the SC and N types. Conventional diagnostic tests did not disclose any difference between the variants in addition to the distinction in their growth patterns on blood agar in semi solid agar and in fluid media Further indications for the identical origin of 3067/66 N NSC and SC come from the results of quantitative transformation assays and DNA buoyant density determinations The close relations of the cell lines are also suggested by their antigenic similarity since previously serological variation has been demonstrated between different strains of *M nonliquefaciens* (14)

The differences in electron microscopical morphology therefore represent a striking variability within the same strain The control examinations of three other strains leave no doubt that there is correlation between the spreading corroding colony type of *M nonliquefaciens* and the occurrence of cells with fimbriae* No previous account has presented fimbriation as typically associated with colony type in this species What could be corresponding structures have been reported as stiff long filaments of 150 Å width in organisms called *Moraxella e g M lwoffi* (22 23) These relatively thick appendages were considered not to be fimbriae but proflagella with a hypothetical causative relationship to gliding motility It was not specifically stated whether the filaments were observed in organisms belonging to genus *Moraxella* in the strict sense (15) which includes *M nonliquefaciens* and other oxidase positive species excluding the oxidase negative *M lwoffi* Fimbriae have been described in other bacterial groups Enterobacteriaceae *Pseudomonas Caulobacter* (10) and *Corynebacterium renale* (24) The SC fimbriae of *M non*

* In this publication the term "fimbriae" (10 19) is used instead of the synonym "pili" (1 2)

quefaciens are probably 60–70 Å in diameter (see Results) Measurements of the width in negatively stained preparations tend to render too low figures (18) *Brinton* (2) found a center to center distance of 63 Å in type 1 fimbriae of *E coli* in negative stain preparations while the diameter of a single filament was 70 Å from other measurements This indicated to him the presence of an interlocking surface fine structure

The observed association between fimbriation and spreading colonies of *M nonliquefaciens* (an SC colony once reached a diameter of 26 mm) and the formation of woolly outgrowths from the stab in semisolid medium may reflect the involvement of fimbriae in some kind of motility It is interesting to find that other bacterial genera with typically spreading colonies *Proteus* and *Pseudomonas* reveal fimbriae with the same order of thickness as our *M nonliquefaciens* (10) The type 1 fimbriae of *E coli* however are characteristic of smooth usually small colonies (1) The circumstance that SC and to some extent NSC cultures tend to form sturdy pellicles on broth surfaces combined with their higher ultimate cell yield indicate that these filaments participate in oxygen uptake as suggested for *Shigella* and *Salmonella* (10 11 12) The constantly lower transformation competence observed for the non fimbriated N form could reflect the role of fimbriae in DNA uptake but may merely be a fortuitous expression of physiological differences between the variants

The relationship of the *M nonliquefaciens* fimbriae regularly found in SC cultures to the sparse filaments in N cultures is unknown Indeed further studies are needed to determine whether the N type filaments are continuous with the cellular bodies Anyhow the N filament fragmentation indicates that they are easily detached The single observation of N filament like structures in an SC culture which also contained SC fimbriae (Fig 12) opens the possibility that this component is shared by the two colony types

The detailed interpretation of the serological absorption experiments are difficult The agglutinins to both native and heated N cells were removed completely by heterologous absorptions as were the agglutinins to heated SC cells Thus all N antigens are shared by the SC type and the thermostable antigens are identical In a few experiments also anti SC agglutinins appeared to be removed by N absorption which is compatible with serological identity between N and SC populations This would conform with electron microscopical data of fragmented filaments [illegible] N (and SC) cultures [illegible] structurally modified SC fimbriae [illegible] often agglutination was [illegible] S cells in N absorbed SC [illegible] favour of an SC specific [illegible] unless the explanation is [illegible] the fimbriated cells are [illegible] glutination Fimbriae [illegible] sociated with adhesiveness [illegible] the SC cells were never [illegible] immune sera we consider [illegible] immunologically specific [illegible] the SC_0 agglutinates corre[illegible] brial agglutination [illegible] loosely floccular which [illegible] fine granules upon shaking [illegible] the specificity appears to be fimbrial The incompleteness of the agglutinations in absorbed sera indicates that only a small and probably varying proportion of the SC cells carry fimbriae with the antigenic determinant in question Quantitative differences in fimbriation of SC cells were observed in the electron microscope (cfr Fig 13) The possibility of *phase variation in thermolabile (fimbrial) SC* antigens is not excluded

Separate fimbrial antigens have been demonstrated in other species in *E coli* by injecting mechanically separated fimbriae (1) and in *Shigella flexneri* similarly to our approach by immunization with fimbriated and absorption with non fimbriated cells (13) In the latter organism (a) crude fimbrial sera reacted to a higher titer with fimbrial phase cells and (b) non fimbrial antisera exhibited higher titers with non fimbriated than with homologous fimbriated phase cells. Our results in *M nonliquefaciens* indicate also consistent

ly higher titers with the SC_o cells than with the N_o cells against SC_o antisera, but no difference seemed to exist between the two cell lines in N_o antisera

The presence of an envelope antigen in *M nonliquefaciens* constitutes a possible explanation of why cells acquire increased agglutinability after heating as was observed here and has been found also by *Haug & Henriksen* (14) The assumption would be that native cells stimulate the production of antibody against the O antigen or perhaps less likely that the envelope antigen which cannot stimulate antibody production after heating (Table 2) is still capable of eliciting agglutination Since the absorption power remains after heating the envelope antigen corresponds to the B-type (17 19) An envelope antigen has not previously been described in *Moraxella*

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OXIDASE POSITIVE BACTERIA IN THE HUMAN NOSE INCIDENCE AND SPECIES DISTRIBUTION, AS DIAGNOSED BY GENETIC TRANSFORMATION

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By diagnostic application of genetic transformation the occurrence of oxidase positive bacteria in the human nose cavity was examined *Neisseria catarrhalis* and *Moraxella nonliquefaciens* were the predominating organisms being equally frequent and representing 87 3 per cent of 111 oxidase positive isolates In part of the study comprising 130 patients *N catarrhalis* was isolated from 16 2 per cent and *M nonliquefaciens* from 17 7 per cent of the nose specimens *M liquefaciens* and *M osloensis* were isolated only occasionally whereas the two species *M phenylpyrouvica* and *M kingii* were not encountered Ten per cent of the isolates belonged to genus *Neisseria in the strict sense* An ecological distinction is thereby indicated between rodshaped moraxellae (*e g M nonliquefaciens*) and false neisseriae (*e g N catarrhalis*) on the one hand and the "true neisseriae" on the other with only the former groups having an important habitat in the nose *N elongata* a rodshaped member of genus *Neisseria* was not observed in this location About 80 per cent of the *N catarrhalis* and *M nonliquefaciens* strains were competent in genetic transformation

The affinities in streptomycin resistance transformation of oxidase positive Gram negative and nonmotile bacteria (moraxellae and neisseriae) form an important basis in discussion of their classification (4 9 10 ... 13 16 17 19) By using this approach in identification the following main problems were sought clarified.

(a) Is the nose cavity an important habitat of false neisseriae (e g *Neisseria catarrhalis*[1]) as well as rodshaped moraxellae and usually not to the same extent as members of genus *Neisseria* in the strict sense ... Previous reports are conflicting as regards the incidence of *N catarrhalis* in the human nasopharynx (3) and there are very few reports specifically concerned with its occurrence in the nose

(b) What is the relative species incidence among oxidase positive rods in this location Henriksen frequently observed *Moraxella duplex* var *nonliquefaciens* in the nose (15) but later this concept was divided into three species entities, the redefined *M nonliquefaciens*, *M osloensis* and *M phenylpyrouvica* (9 10)

Received 29 vi 70

[1]) In this report the classical designation of this species is used (12)

MATERIALS AND METHODS

Origin and cultivation of specimens The material consisted exclusively of routine nose specimens from outpatients mostly with various ear nose throat ailments Sampling was performed by means of cotton swabs from the whole length of the nose cavity trying not to touch the walls of *vestibulum* The cultures were made on blood agar plates as a rule by the examining oto-rhino laryngologists and immediately brought to the bacteriological laboratory The plates were streaked with *Staphylococcus aureus* and incubation performed for two days at 35 C in a humid atmosphere The first part of the investigation took place on predetermined days during August September and October 1966 From all oxidase positive cultures one colony was isolated and lyophilized If the colony characteristics indicated that neisseriae and moraxellae were simultaneously present a colony of each was picked The total number of examined specimens during this period is not available The last part of the study was performed during January and February 1970 also on preselected days The number of nose specimens examined during this latter period was 130 Specimens with swarming *Proteus* were excluded

Diagnostic procedures The biochemical and cultural tests employed have been described in previous reports (4 10 11) Streptomycin resistance transformation was performed according to the short term (20 min) or long term (continuous) DNA exposure techniques used in those studies) The usual procedure was as follows Oxidase positive rods which did not liquefy serum were exposed simultaneously to stock DNAs from streptomycin resistant mutants of *M nonliquefaciens* and *M osloensis* In case the parallel with the former DNA revealed a significant number of transformants whereas the other parallel was negative or almost so (mutation to streptomycin resistance is extremely infrequent in *M nonliquefaciens* and most other moraxellae contrary to the situation in neisseriae) the diagnosis *M nonliquefaciens* was considered verified In previous sensitive transformation experiments with long term DNA exposure no genetic compatibility was observed between this species and *M phenylpyrouvica* *M lacunata*? *M kingii* or *N elongata* (4 10 11 16) Approximately 75 per cent of the *M nonliquefaciens* strains could be diagnosed in this simple manner by the use of long term DNA exposure in case short term exposure was not sufficient to give numerous transformants Analogously 85 per cent of the *N catarrhalis* strains were diagnosed by the use of three DNAs from *N catarrhalis* Ne 11 *N catarrhalis* NCTC 4103 and *N meningitidis* Diagnostic for typical *N catarrhalis* are thousands of transformants with the first mentioned DNA about 1/100 or less of that with DNA from the second [illegible] str [illegible] (4 [illegible] 4) and none with *N meningitidis* DNA [illegible] ing strains of *M nonliquefaciens* and *N* [illegible] and those belonging to [illegible] identified by the ordinary q[illegible] procedure [illegible] streptomycin resistant mutants [illegible] study and comparison [illegible] of DNA from previously identified [illegible] recipients of the latter More[illegible] autologous transformation [illegible] a sufficient requirement [illegible]

RESULTS

A total of 111 strains [illegible] Gram negative and immotile [illegible] isolated Of these 59 were [illegible] material In the 1970 study [illegible] from the nose, such organisms [illegible] ered in 48 cases i e, 36 9 [illegible] cases both a coccal (*N* [illegible] and a rodshaped strain (*M nonliquefaciens*) [illegible] recovered

In the 107 positive specimens [illegible] oxidase positive organisms were found in [illegible] mostly in dense culture, whereas in the remainder one observed simultaneous occurrence of *Haemophilus* 4 *Streptococcus pneumoniae* 11 *Str pyogenes* 1 *Str viridans* or non haemolytic streptococci 3 *Staphylococcus aureus* 15 *Staph epidermidis* 18 or corynebacteria 23 cases In 82 cases without oxidase positives a slightly higher incidence of all the mentioned oxidase negative species was observed (the 1970 material)

The cases which revealed only oxidase positive organisms were compared with those from which *Haemophilus* and *Streptococcus pneumoniae* were isolated and with the cases where only corynebacteria were found All four groups appeared random as regards clinical diagnoses with *otitis media acuta* and *rhinosinusitis* as the most frequent

As shown in Table 1 46 8 per cent of the oxidase positive isolates belonged to *N catarrhalis* and 40 5 per cent to *M nonliquefaciens* In the part of the material for which the total number of examined specimens is known *N catarrhalis* was isolated from 16 2 per cent of the patients and *M nonlique*

faciens from 17 7 per cent None of the isolated strains corresponded genetically to the deviating *N catarrhalis* strain NCTC 4103 The isolates of *M liquefaciens M osloensis*, *N meningitidis* and *N flava* comprise only 0 9, 1 8 2 7 and 2 7 per cent of the total number of oxidase positive isolates, respectively The remaining five strains were related to *N meningitidis* and/or to *N flava* in transformation but did not show identity reactions with them They showed no genetic compatibility with *N catarrhalis* in sensitive tests Due to the absence of a satisfactory genetic classification system for genus *Neisseria* further genetic identification attempts were not made with these isolates

TABLE 1 *Species Distribution and Incidence of Oxidase Positive Bacteria in the Human Nose*

Species	Number of oxidase positive isolates	Per cent of all oxidase positives	Per cent incidence in 130 of the specimens
*N catarrhalis**	52	46 8	16 ?
M nonliquefaciens	45	40 5	17 7
M liquefaciens	1	0 9	
M osloensis	2	1 8	
N meningitidis	3	2 7	
N flava	3	2 7	
Unidentified neisseriae	5	4 5	

M = *Moraxella* N = *Neisseria*

* In this publication the classical designation of this species is used (12)

The morphology and behaviour in conventional biochemical and cultural tests of these genetic entities generally corresponded to descriptions of the respective species (3 4 9, 12) A few observations will be mentioned The isolates of *N catarrhalis* all had a friable colony texture (not seen in *M nonliquefaciens* of this material), and all except one reduced nitrate to nitrite, contrary to the consistent absence of nitrite reaction in the isolates of *N meningitidis N flava* and the strains genetically related to them Of the latter five isolates two saccharolytic strains died before completion of the examination and the remaining three were different from known species in one or more reactions All except one of the *M nonliquefaciens* strains produced nitrite from nitrate They uniformly failed to grow in Hugh & Leifson's medium contrary to the two isolates of *M osloensis* The latter two strains both grew weakly in Koser's citrate medium, contrary to the *M nonliquefaciens* strains This growth was not significantly inferior to the cultural density obtained when citrate was replaced by acetate in the same base The phenylalanine reaction was negative with all the oxidase positive isolates

DISCUSSION

The results show that the human nose is as frequently inhabited by *N catarrhalis* as by rodshaped moraxellae The incidence of each appears somewhat higher than the value found for *M duplex* var *nonliquefaciens* by *Henriksen* in 1958 (15) in the same kind of specimens and patients (16–20 per cent as compared with 11 per cent) Other oxidase positive species occur much less frequently including those allocated to genus *Neisseria* (true neisseriae) as defined by *Henriksen* & *Bovre* (17)

The high incidence of *N catarrhalis* found here corresponds with the study of Arkwright in 1907 (1), showing the occurrence of *N catarrhalis* in about 35 per cent of normal and catarrhal noses and with other reports early in the century (see ref no 3) However the present results are in apparent conflict with the relatively recent and extensive studies by Berger and others cited by him (3), showing that this species occurs in less than one per cent of nasopharyngeal samples and that true neisseriae are much more frequent These differences could possibly be explained by the existence of an ecological distinction between false neisseriae having their habitat mainly in the nose and the true neisseriae occurring preferentially in the pharynx That the nose habitat is shared by some rodshaped moraxellae is interesting in view of the findings in genetic transformation (4),

DNA base determination (7) and RNA DNA hybridization (5) that false neisseriae and some rodshaped moraxellae are relatively closely related and clearly distinct from the usually saccharolytic or pigmented true neisseriae

The study clearly shows that *M nonlique faciens* as recently defined (9), is the main contributor to the flora of rodshaped mora xellae in the nose cavity *M liquefaciens* (*M lacunata* 17) is rarely occurring as also ob served previously (15) *M osloensis* which has been isolated from a multitude of sources is not frequently met with in the nose since it was encountered only twice *M phenyl pyrouvica* (10) *M urethralis* (19) or *M kingii* (16) were not observed neither was *N elongata* (11) a rodshaped member of genus *Neisseria*

With a genetic diagnosis as basis it is pos sible to discuss the differential value of some conventional diagnostic tests The isolates of *N catarrhalis* were always of a characteristic ally friable colony consistency and as a rule reduced nitrate to nitrite This would usually permit the diagnosis to be made by simple means, with high accuracy It was previously found that *M nonliquefaciens* also reduces nitrate to nitrite (4) contrary to the finding of *Baumann et al* (2) All the present isolates except one gave a positive nitrite reaction when the medium was supplemented with 10 per cent horse serum (4) *M osloensis* is de scribed as growing weakly in citrate media without alkalization (4 9) This was verified with the two new isolates of this species Other investigators have observed that only 0–10 per cent of *M osloensis* strains grow in citrate media when several passages are required whe eas growth in acetate medium is a re gula finding (2 *H Lautrop* personal com mun cation) The strains of *M osloensis* also differed fro[illegible] *M nonliquefaciens* strains by growir[illegible] Hugh & Leifson s medium which was [illegible] pointed out as distinc tive (9) It [illegible]portant to note that no positive phe[illegible] r action was encount ered in the a[illegible] an organism behaving genetically as [illegible] *pyrouvica*

It has been demonstrated how genetic trans formation can be used in relatively extensive diagnostic work on bacteria for which the conventional tests are few and need genetic evaluation In most cases a very simple proce dure of transformation was applicable re quiring only a limited set of standard DNAs and no new DNA extraction The success of this modified technique can be ascribed to the facts that (a) the interspecies affinity is low between the most common rodshaped oxidase positive species in man as also between *N catarrhalis* and the true genus *Neisseria* and that (b) the frequency of strains com petent in genetic transformation is very high in these microbes (80 per cent or more) It was observed that the competence often de clined during serial passages on blood agar, as described separately for some of the *M non liquefaciens* strains (8) Corroding and spread ing colonies (18), which were often observed in the *M nonliquefaciens* strains when partic ularly looked for by prolonged incubation in the humid atmosphere are associated with the occurrence of fimbriated cells and compe tence (6 8) This permitted conservation of the competence by selective subcultivation If this method had been used extensively the percentage of observed competent *M non liquefaciens* strains would probably have been even higher A morphological trait correlated with competence has not yet been observed in *N catarrhalis*

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faciens from 17 7 per cent None of the isolated strains corresponded genetically to the deviating *N catarrhalis* strain NCTC 4103 The isolates of *M liquefaciens*, *M osloensis* *N meningitidis* and *N flava* comprise only 0 9, 1 8 2 7 and 2 7 per cent of the total number of oxidase positive isolates, respectively The remaining five strains were related to *N meningitidis* and/or to *N flava* in transformation but did not show identity reactions with them They showed no genetic compatibility with *N catarrhalis* in sensitive tests Due to the absence of a satisfactory genetic classification system for genus *Neisseria* further genetic identification attempts were not made with these isolates.

TABLE 1 *Species Distribution and Incidence of Oxidase Positive Bacteria in the Human Nose*

Species	Number of oxidase positive isolates	Per cent of all oxidase positives	Per cent incidence in 130 of the specimens
*N catarrhalis**	52	46 8	16 2
M nonliquefaciens	45	40 5	17 7
M liquefaciens	1	0 9	
M osloensis	2	1 8	
N meningitidis	3	2 7	
N flava	3	2 7	
Unidentified neisseriae	5	4 5	

M = *Moraxella* *N* = *Neisseria*
* In this publication the classical designation of is species is used (12)

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